UNIVERZITA KARLOVA
3. LÉKAŘSKÁ FAKULTA

DIZERTAČNÍ PRÁCE

MECHANIZMY INDUKCE A INHIBICE APOPTÓZY MASTNÝMI KYSELINAMI U PANKREATICKÝCH β-BUNĚK

RNDr. JAN ŠRÁMEK

ŠKOLITEL: **PROF. RNDR. JAN KOVÁŘ, DRSC.**
STUDIJNÍ PROGRAM: **MOLEKULÁRNÍ A BUNĚČNÁ BIOLOGIE, GENETIKA A VIROLOGIE**

ÚSTAV BIOCHEMIE, BUNĚČNÉ A MOLEKULÁRNÍ BIOLOGIE
ODDĚLENÍ BUNĚČNÉ A MOLEKULÁRNÍ BIOLOGIE

PRAHA 2018

PROHLÁŠENÍ

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem řádně uvedl a citoval všechny použité prameny a literaturu. Současně prohlašuji, že práce nebyla využita k získání jiného nebo stejného titulu. Tato práce vznikla pod vedením prof. Jana Kováře na Oddělení buněčné a molekulární biologie, 3. lékařské fakulty, Univerzity Karlovy.

Práce byla finančně podporována grantem MSM 0021620814 Ministerstva školství, mládeže a tělovýchovy České republiky, granty SVV-2010-260704, SVV-2011-262706, GAUK 1270213 a projekty UNCE 204015, PRVOUK P31, PROGRES Q36 Univerzity Karlovy.

Souhlasím s trvalým uložením elektronické verze mé práce v databázi systému meziuniverzitního projektu Theses.cz za účelem soustavné kontroly podobnosti kvalifikačních prací.

Praha, 29. 5. 2018

RNDr. Jan Šrámek

Identifikační záznam:

ŠRÁMEK, Jan. *Mechanizmy indukce a inhibice apoptózy mastnými kyselinami u pankreatických β-buněk*. [*Mechanisms of apoptosis induction and inhibition by fatty acids in pancreatic β-cells*]. Praha, 2018. 139 s. Dizertační práce. Univerzita Karlova, 3. lékařská fakulta, Ústav biochemie, buněčné a molekulární biologie, Oddělení buněčné a molekulární biologie 3. LF UK. Vedoucí práce prof. RNDr. Jan Kovář, DrSc.

Klíčová slova: pankreatické β-buňky, apoptóza, mastné kyseliny, hypoxie, DMT2, signální dráhy

Key words: pancreatic β-cells, apoptosis, fatty acids, hypoxia, DMT2, signaling pathways

PODĚKOVÁNÍ

Na tomto místě bych chtěl poděkovat všem, kteří mi nějakým způsobem pomáhali či mě podporovali při tvorbě této práce. Zejména pak svému školiteli prof. Janu Kovářovi za vstřícnost, trpělivost, cenné rady, připomínky a odborné vedení obecně, díky kterému mohla být tato práce dokončena.

OBSAH

1. SEZNAM ZKRATEK

AIF	apoptosis-inducing factor
AMPK	adenosine monophosphate-activated protein kinase
Apaf-1	apoptotic protease activating factor 1
AP-1	activator protein-1
ASK1	apoptosis signal-regulating kinase 1
ATF2	activating transcription factor 2
ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
ATF6f	activating transcription factor 6 fragment
ATP	adenosine triphosphate
Bad	Bcl-2-associated death promoter
Bax	Bcl-2-associated X protein
Bcl-2	B-cell CLL/lymphoma-2
Bcl-xL	B-cell leukemia/lymphoma-extra large
Bid	BH3-interacting domain
BiP	immunoglobulin heavy chain-binding protein
BNIP3	Bcl-2/adenovirus E1B 19kDa interacting protein 3
BSA	bovine serum albumin (bovinní sérový albumin)
CAD	caspase-activated deoxyribonuclease
CARD	caspase recruitment domain
CHOP	C/EBP-homologous protein
CD36	cluster of differentiation 36
c-FLIP	cellular FLICE-like inhibitory protein
DISC	death-inducing signaling complex
DMT1	diabetes mellitus typu 1
DMT2	diabetes mellitus typu 2
DNA	deoxyribonucleic acid
DR3	death receptor 3
eIF2α	eukaryotic initiation factor 2α
eIF-4E	eukaryotic translation initiation factor 4E
ERK1/2	extracellular signal-regulated kinase 1/2
ER	endoplazmatické retikulum
ERAD	endoplasmic reticulum-associated degradation
FADD	Fas-associated death domain
FAT	fatty acid translocase
FATP	fatty acid transport protein
GADD34	growth arrest and DNA damage 34
GLUT1	glucose transporter 1
GTP	guanosine triphophate
Grp78	glucose-regulated protein 78
HIF	hypoxia-inducible factor
HtrA2/Omi	high temperature requirement protein A2
IAP	inhibitors of apoptotic proteins
iNOS	inducible nitric oxide synthase
IRE1α	inositol-requiring protein 1α
IUF1	insulin upstream factor 1
JNK	c-Jun N-terminal kinase
LC-CoA	long-chain acyl-coenzyme A
LDH	lactat dehydrogenase
MAPK	mitogen-activated protein kinase
MAPKAPK-2	MAP kinase-activated protein kinase 2

MAP3Ks	mitogen-activated protein kinase kinase kinase
MEK1/2	mitogen-activated protein kinase/ERK kinase 1/2
MK	mastné kyseliny
MKK	mitogen-activated protein kinase kinase
MLKs	mixed-lineage kinases
MNKs	MAP kinase signal-integrating kinases
MSK1/2	mitogen- and stress-activated protein kinase 1/2
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa B
NO	nitric oxide
NOS	nitric oxide synthase
OA	oleic acid (kyselina olejová)
PA	palmitic acid (kyselina palmitová)
PAK	p21-activated protein kinase
PBS	programovaná buněčná smrt
PDK1	phosphoinositide-dependent protein kinase
PERK	protein kinase RNA-like endoplasmic reticulum kinase
PIDD	p53-induced protein with a death domain
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
POA	palmitoleic acid (kyselina palmitolejová)
PP2a	protein phosphatase 2a
PTP	permeability transition pore
Rac1	Ras-related C3 botulinum toxin substrate 1
RAIDD	RIP-associated protein with a death domain
Ras	rat sarcoma
ROS	reactive oxygen species
RSKs	p90 ribosomal S6 kinases
RTK	receptor tyrosine kinase
SA	stearic acid (kyselina stearová)
Smac/DIABLO	second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with low pI
siRNA	small interfering RNA
SOS	son of sevenless
SREBP	sterol-regulatory element-binding protein
Syk	spleen tyrosine kinase
TAB1	TAK1-binding protein
TAK1	transforming growth factor-β-activated protein kinase 1
TNFR1	tumour necrosis factor receptor 1
TNF-α	tumour necrosis factor α
TRADD	TNFR-associated via death domain
TRAF2	TNFR-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
UPR	unfolded protein response
WHO	World Health Organisation
XBP1	X-box binding protein 1
XBP1s	spliced X-box binding protein 1
ZDF	Zucker diabetic fatty

2. ABSTRAKT

Diabetes mellitus 2. typu (DMT2) dnes představuje z hlediska incidence a ekonomických dopadů jednu z nejvýznamnějších metabolických chorob. Mezi hlavní příčiny tohoto onemocnění patří ztráta funkce a viability pankreatických β-buněk v důsledku působení zvýšených hladin nasycených mastných kyselin (MK). Nenasycené MK jsou β-buňkami lépe tolerovány. Dokonce jsou schopny inhibovat poškozující účinky nasycených MK. Molekulární mechanizmy indukce apoptózy pankreatických β-buněk nasycenými MK stejně jako mechanizmy inhibice této indukce nenasycenými MK jsou dosud nejasné. Hlavním cílem této práce bylo přispět k objasnění těchto mechanizmů.

U lidské linie pankreatických β-buněk NES2Y jsme prokázali: (1) Aktivace kaspázy 2 působením nasycené kyseliny stearové (SA) v koncentraci indukující apoptózu (1 mM) není pro proces indukce apoptózy zásadní. Tato kaspáza však moduluje dráhy stresu endoplazmatického retikula (ER) indukované působením SA. (2) SA (1 mM) aktivuje p38 MAPK signální dráhu a inhibuje ERK signální dráhu. Inhibice ERK signální dráhy je pravděpodobně důsledkem aktivace p38 MAPK dráhy. Avšak p38 MAPK nejspíše není pro indukci apoptózy působením SA klíčová. Nenasycená kyselina olejová (OA, 0,2 mM) je schopná inhibovat účinky SA na uvedené signální dráhy. Sama o sobě ovlivňuje zmíněné signální dráhy pouze minimálně. (3) SA (1 mM) aktivuje dráhy stresu ER, tj. dráhy IRE1α, PERK a ATF6. OA (0,2 mM) inhibuje účinky SA na dráhy IRE1α a PERK a sama o sobě ovlivňuje aktivaci těchto drah pouze minimálně. Vliv OA na ATF6 dráhu nebyl zjišťován. JNK kináza, obdobně jako kaspáza 2, není pro proces indukce apoptózy působením SA zásadní, ale moduluje dráhy stresu ER aktivované touto MK. (4) Místo navození proapoptotické signalizace nasycenými MK, stejně jako místo inhibiční intervence nenasycených MK do mechanizmů indukce apoptózy nasycenými MK, se nalézá „upstream“ od studovaných signálních drah. Pravděpodobně se nachází již na plazmatické membráně buněk. (5) Hypoxie zesiluje proapoptotický efekt SA (1 mM) nejspíše prostřednictvím zvýšení signalizace stresu ER. Hypoxie také snižuje protektivní efekt OA (0,2 mM) na proapoptotický účinek SA a to takovým způsobem, že OA již není schopna blokovat indukci apoptózy β-buněk vyvolanou působením SA. Hypoxie samotná má na β-buňky relativně slabý poškozující vliv. Hypoxie tedy může představovat klíčový faktor rozhodující o přežívání/smrti pankreatických β-buněk v přítomnosti MK a tedy v důsledku toho i potenciálně o vzniku DMT2.

Naše výsledky přispěly k porozumění mechanizmů, kterými nasycené MK indukují apoptózu pankreatických β-buněk, a mechanizmů inhibice této indukce nenasycenými MK. Výsledky také přispěly k objasnění vlivu hypoxie na indukci apoptózy nasycenými MK a na inhibici této indukce nenasycenými MK u β-buněk. Tyto poznatky by mohly být významné při hledání nových postupů terapie DMT2 zaměřených na zachování funkce a viability pankreatických β buněk.

3. ABSTRACT

Recently, diabetes mellitus type 2 (DMT2) represents one of the most important metabolic diseases according to its incidence and economic impacts. One of the main reasons of this diesease is loss of function and viability of pancreatic β-cells due to the effect of increased levels of saturated fatty acids (FAs). Unsaturated FAs are better tolerated by β-cells. They are even capable of inhibiting detrimental effects of saturated FAs. Molecular mechanisms of apoptosis induction in pancreatic β-cells by saturated FAs as well as mechanisms of inhibition of this induction by unsaturated FAs are not completely elucidated. The main aim of this study was to contribute to elucidation of these mechanisms.

Concerning human pancreatic β-cell line NES2Y we demonstarted: (1) Activation of caspase-2 by stearic acid (SA), in apoptosis inducing concentration (1 mM), is not crucial for the process of apoptosis induction. However, this caspase modulates SA-induced endoplasmic reticulum (ER) stress pathways. (2) SA (1 mM) activates the p38 MAPK signaling pathway and inhibits the ERK signaling pathway. Inhibition of the ERK signaling pathway is probably a consequance of the p38 MAPK pathway activation. However, p38 MAPK is not very likely crutial for the apoptosis induction by SA. Unsaturated oleic acid (OA, 0.2 mM) is able to inhibit the effects of SA on mentioned signalling pathways. OA itself has only minimal effect on these signaling pathways. (3) SA (1 mM) activates ER stress pathways, i.e. IRE1α, PERK and ATF6 pathways. OA (0.2 mM) is able to inhibit the effects of SA on IRE1α and PERK pathways and itself has only minimal effect on the activation of these pathways. Effect of OA on the ATF6 pathway was not tested. JNK kinase, similarly as caspase-2, is not crucial for the process of apoptosis induction by SA, but it modulates ER stress pathways activated by SA. (4) The point of induction of pro-apoptotic signaling by saturated FAs, as well as the point of inhibitory intervention of unsaturated FAs into mechanisms of apoptosis induction by saturated FAs, is located upstream of studied signaling pathways. It is probably located on the plasma membrane of cells. (5) Hypoxia potentiates pro-apoptotic effect of SA (1 mM), probably via increased ER stress signaling. Hypoxia also decreases protective effect of OA (0.2 mM) on pro-apoptotic effect of SA in such way that OA is not able to block the induction of apoptosis of β-cells due to SA effect anymore. Hypoxia alone has relatively weak detrimental effect on β-cells. Thus, hypoxia can represent a key factor which is decisive for survival/death of pancreatic β-cells in the presence of FAs and thus, as a consequence, also potentially decisive for the development of DMT2.

Our results contribute to understanding of mechanisms by which saturated FAs induce apoptosis of pancreatic β-cells and mechanisms of inhibition of this induction by unsaturated FAs. Results also contribute to eluciodation of the effect of hypoxia on apoptosis induction by saturated FAs and on inhibition of this induction by unsaturated FAs in β-cells. These findings could be important in the search for new therapeutic approaches of DMT2 focused on maintaining function and viability of pancreatic β-cells.

4. CÍLE PRÁCE

Diabetes mellitus 2. typu dnes představuje z hlediska incidence a ekonomických dopadů jednu z nejvýznamnějších metabolických chorob. Mezi hlavní příčiny tohoto onemocnění patří ztráta funkce a viability pankreatických β-buněk v důsledku působení zvýšených hladin nasycených mastných kyselin (MK). Nenasycené MK jsou β-buňkami lépe tolerovány. Dokonce jsou schopny inhibovat poškozující účinek nasycených MK (Fürstová et al. 2008, Němcová-Fürstová et al. 2011). Molekulární mechanizmy indukce apoptózy pankreatických β-buněk nasycenými MK a mechanizmy inhibice této indukce nenasycenými MK nejsou dosud plně objasněny. Určitou úlohu zde pravděpodobně hrají relevantní signální dráhy včetně drah stresu endoplazmatického retikula (ER). Hlavním cílem této práce bylo přispět k objasnění molekulárních mechanizmů indukce apoptózy nasycenými MK a mechanizmů inhibice této indukce nenasycenými MK u lidských pankreatických β-buněk.

Konkrétně jsme se zaměřili na:

1. úlohu kaspázy 2 v indukci apoptózy nasycenými MK
2. úlohu p38 MAPK a ERK signálních drah v indukci apoptózy nasycenými MK a v inhibici této indukce nenasycenými MK
3. úlohu drah stresu ER v indukci apoptózy nasycenými MK a v inhibici této indukce nenasycenými MK
4. určení místa navození proapoptotické signalizace nasycenými MK a určení místa inhibiční intervence nenasycených MK do mechanizmů indukce apoptózy nasycenými MK
5. vliv hypoxie na indukci apoptózy nasycenými MK a na inhibici této indukce nenasycenými MK

5. LITERÁRNÍ PŘEHLED

5.1. Pankreatické β-buňky

5.1.1. Langerhansovy ostrůvky a pankreatické β-buňky

Savčí pankreas se skládá z exokrinní a endokrinní tkáně. Endokrinní tkáň reprezentují Langerhansovy ostrůvky, které se zásadním způsobem podílí na udržování požadovaných hladin živin v krvi. Tyto ostrůvky představují asi 2 % váhy pankreatu a jejich počet se pohybuje u dospělého člověka mezi 1 a 2 miliony (Kulkarni 2004, Meier et al. 2008). Jsou tvořeny hlavně β-buňkami produkujícími inzulín (65–90 % buněk ostrůvků, asi 3000 na ostrůvek), (Butler et al. 2003, Ritzel et al. 2007), které tvoří jádro ostrůvku. Dále α-buňkami produkujícími glukagon (15–20 %), δ-buňkami produkujícími somatostatin (3–10 %) a PP-buňkami produkující pankreatický polypeptid (1 %). Ty jsou pak většinou lokalizovány na povrchu ostrůvku (Collombat et al. 2010).

5.1.2. Diabetes mellitus 2. typu a jeho patogeneze

Diabetes mellitus je metabolické onemocnění, které podle údajů Světové zdravotnické organizace (WHO), (http://www.who.int/mediacentre/factsheets/fs312/en/) postihuje téměř 350 milionů lidí na celém světě. Hlavním projevem této choroby je chronická hyperglykémie, která vzniká v důsledku nedostatečného množství inzulínu k pokrytí potřeb organizmu. Rozlišujeme dva základní typy tohoto onemocnění – diabetes mellitus typu 1 a 2 (DMT1 a DMT2). 90 % pacientů, kteří trpí diabetem, mají diagnostikován DMT2 (WHO).

Jednou z hlavních obecných příčin rozvoje DMT2 je moderní životní styl populace, který vede k obezitě. Především se jedná o chronicky zvýšený příjem potravy často nevhodné skladby v kombinaci s nízkou fyzickou aktivitou vedoucí k nadváze a posléze obezitě. Přes 40 % pacientů s DMT2 trpí nadváhou či obezitou (WHO). Nárůst hmotnosti je spojen se zvýšením požadavku na produkci inzulínu. Kromě toho bylo prokázáno, že obezita má spojitost se vznikem inzulínové rezistence (Kahn et al. 1993). Inzulinová rezistence se rozvíjí již několik let před stanovením diagnózy DMT2. Projevuje se sníženou stimulací transportu glukózy ve svalech a tukové tkáni a zvýšenou produkcí inzulínu. Zvýšená produkce inzulínu představuje snahu organizmu kompenzovat nedostatečný efekt inzulínu v rezistentních tkáních. V důsledku inzulínové rezistence je tedy požadavek na produkci inzulínu ještě umocněn. Když nejsou β-buňky schopny kompenzovat relativní nedostatek inzulínu, dochází k jejich vyčerpání, dysfunkci a

následné apoptóze, což vyústí v DMT2. V době, kdy je DMT2 diagnostikován, bývá funkce β-buněk snížena zhruba na polovinu (Pratley & Weyer 2001, Defronzo 2009). Vztah mezi obezitou, inzulínovou rezistencí a DMT2 byl jednoznačně prokázán (Steyn et al. 2004, Kahn et al. 2006). Snížení funkce a počtu β-buněk je dnes přijímáno jako hlavní příčina (na buněčné úrovni) vzniku a rozvoje DMT2 (Donath et al. 2005, Maedler 2008, Thomas et al. 2009, Defronzo 2010, Poitout et al. 2010, Fonseca et al. 2011). Vedle apoptózy β-buněk v důsledku jejich vyčerpání se v patogenezi DMT2 významným způsobem uplatňují ještě další faktory. Obézní lidé mají chronicky zvýšenou hladinu mastných kyselin (Kahn et al. 2006) v krvi. Tento faktor se ukazuje být, spolu se zvýšenou koncentrací glukózy v krvi, zásadní příčinou dysfunkce a následné apoptózy pankreatických β-buněk (viz kapitola 5.3.2, str. 19).

5.1.3. Experimentální modely používané pro studium procesů asociovaných s diabetem typu 2

Experimentální modely, které jsou běžně využívány k studiu procesů asociovaných s DMT2 představují, podobně jako u jiných typů metabolických či jiných onemocnění, vzhledem k etickým zábranám především zvířecí modely a buněčné linie. Jsou také využívány izolované Langerhansovy ostrůvky především zvířecího, ale i lidského původu. Nicméně jejich využití je výrazně limitováno jejich dostupností, obtížností izolace a někdy též legislativou.

Jako zvířecí modely jsou nejčastěji využívány hlodavčí modelové organizmy a to vzhledem k jejich malé velikosti, rychlému množení, ekonomické úspornosti, atp. DMT2 může u těchto modelových organizmů vzniknout nebo být vyvolán několika způsoby: (1) spontánně, tj. na základě přítomnosti autosomálně recesivní mutace v genu kódujícím leptin (*ob/ob* či *db/db* myši, *fa/fa* krysy), (Zhang et al. 1994, Lee et al. 1996, Phillips et al. 1996); (2) vhodnou dietou (myší kmen C57BL/6J, pískomil tlustý) či medikací (Surwit et al. 1988, Shafrir & Ziv 1998, Srinivasan & Ramarao 2007); (3) chirurgickým zásahem (pankreatomií), (Hinke et al. 2016) nebo (4) pomocí genových modifikací (přehledně v Kadowaki 2000).

Pro výzkum DMT2 se dnes používá řada linií pankreatických β-buněk (přehledně v Skelin et al. 2010). Mezi nejvíce používané nebo významné vzhledem k tématu této práce patří linie RIN, BRIN, INS-1, HIT, βTC, MIN6 a NES2Y. Buněčná linie NES2Y, kterou jsme používali v našich experimentech, byla odvozena z pacienta s dětskou

chronickou hyperinzulinemickou hypoglykémií (nasidioblastóza). Tyto buňky v důsledku ztráty funkce adenosin trifosfát (ATP)-regulovaného draselného kanálu kontinuálně vylučují inzulín (Macfarlane et al. 1997). Sekrece inzulínu tedy není regulována glukózou.

5.2. Apoptóza

5.2.1. Apoptóza a její biologická úloha

Termín apoptóza (apoptosis) byl prvně použit v dnes již klasické práci autorského kolektivu Kerra, Wyllieho a Currie z roku 1972 k popisu morfologicky specifického typu buněčné smrti (Kerr et al. 1972) a pochází ze složeniny dvou řeckých slov "apo" a "ptosis" znamenající odpadnout od něčeho. Molekulární průběh tohoto procesu byl poprvé podrobně popsán na základě studia 131 odumírajících buněk hlísta *Caenorhabditis elegans* v průběhu jeho determinované ontogeneze (Bursch et al. 2000, Kinchen & Hengartner 2005, Kumar 2007). Ta je u jednotlivých druhů hlístů vždy totožná. Na základě těchto prací byla následně apoptóza uznána jako jeden z typů tzv. programované buněčné smrti (PBS). PBS je buněčná smrt, která představuje geneticky determinovanou eliminaci buněk realizovanou na základě specifických signálů ve vhodném čase a místě podle určitých mechanizmů. Každá buňka v mnohobuněčném organismu tedy nese geneticky kódovanou informaci pro realizaci vlastní sebedestrukce. Jak již bylo zmíněno výše, apoptóza nepředstavuje jediný známý typ PBS, ale rozeznáváme ještě minimálně jeden další a sice autofagii. Ta je někdy označována jako PBS typu II (Debnath et al. 2005, Tsujimoto & Shimizu 2005). Je možné, že oba uvedené typy PBS jsou propojeny, kdy autofagie může vyústit v apoptózu (Piacentini et al. 2003)

Vedle PBS existuje ještě buněčná smrt označovaná jako nekróza. Ta nastává v případech, kdy jsou vnější podmínky neslučitelné s přežitím buňky, kdy se buňka na procesu buněčné smrti aktivně nepodílí. Uvedené dva základní typy buněčné smrti je možné odlišit na základě biochemické a morfologické charakteristiky (přehledně v Elmore 2007). Nové poznatky časem ukázaly, že toto základní rozdělení buněčné smrti na PBS a nekrózu je příliš zjednodušené. Byla totiž dokumentována řada případů, kdy uvedené biochemické a morfologické charakteristiky nespadají ani do jedné z uvedených klasifikací či existují jakési přechodové formy (Formigli et al., 2000, Sperandio et al. 2000, Bursch 2004, Fietta 2006, Christofferson & Yuan 2010).

Bylo zjištěno, že apoptóza hraje u mnohobuněčných organismů významnou úlohu v mnoha fyziologických a patofyziologických procesech. Apoptóza má úlohu v normální

fyziologii již v průběhu ontogenese, kdy se tento proces zásadním způsobem podílí na morfogenesi tkání. Jde například o morfogenezi prstů, mozku či úplné nebo částečné regresi již nepotřebných orgánů v určité etapě vývoje jedince jako je například ocas žab (Jacobson et al. 1997, Meier et al. 2000, Nijhawan et al. 2000). V dospělosti se pak apoptóza uplatňuje například při regresi mléčných žláz po odstavení nebo při folikulární atrézii v post-ovulačním období (Tilly 1991, Lund et al. 1996). Dále je pak, vzhledem ke své roli při pozitivní a negativní selekci lymfocytů, zásadní pro správnou funkci imunitního systému (Opferman & Korsmeyer 2003). Nelze opomenout úlohu apoptózy při eliminaci buněk napadených patogeny či obecně různým způsobem postižených buněk (narušení funkce či proliferační aktivity), jejichž existence je pro organizmus potenciálně nebezpečná. Důležitou roli má i v procesu hojení ran (Greenhalgh 1998). Patrně nejvýznamnější úlohou apoptózy v dospělém organizmu je udržování tkáňové homeostáze (přehledně v Elmore 2007).

5.2.2. Molekulární mechanizmy indukce a exekuce apoptózy

Apoptóza může být vyvolána různými stimuly, například ligandy pro specializované receptory, tzv. "receptory smrti", nedostatkem stimulačních faktorů nebo nutričních složek, působením γ záření nebo působením chemických látek (Cornelis et al. 2005, Ehrlichová et al. 2005, Afshar et al. 2006, Kralova et al. 2006, Lavrik et al. 2006, Matsubara et al. 2006). Důležitou skupinou proteinů iniciace a exekuce apoptózy jsou kaspázy. Jsou to cysteinové proteázy štěpící své substráty za aspartátem. Mezi iniciační kaspázy, tedy kaspázy uplatňující se v iniciační fázi apoptózy, se řadí kaspázy 2, 8, 9 a 10. Mezi exekuční kaspázy patří kaspáza 3, 6 a 7. Kaspázy 1, 4 a 5 se pak účastní zánětlivých reakcí (Cohen 1997, Rai et al. 2005). Iniciační kaspázy jsou aktivovány ve specifických komplexech označovaných jako PIDDozóm (kaspáza 2), DISC (death-inducing signaling complex), (kaspáza 8 a 10) a apoptozóm (kaspáza 9), (Kischkel et al. 1995, Adams & Cory 2002 Tinel & Tschopp 2004). Hlavními regulátory aktivity kaspáz jsou proteiny rodiny IAP (inhibitors of apoptosis proteins), které kaspázy inhibují vazbou do katalytického místa nebo vyvazují jejich adaptorové proteiny (Deveraux et al. 1997, Roy et al. 1997, Pop & Salvesen 2009).

5.2.2.1. Molekulární mechanizmy indukce apoptózy

Molekulární mechanizmy indukce apoptózy představují vysoce komplexní a sofistikovaný sled událostí, v rámci kterého lze rozlišit dvě základní signální dráhy: tzv. "vnější" a "vnitřní" dráhu.

Vnější dráha (označovaná též jako receptorová) je zahajována na buněčném povrchu prostřednictvím povrchových receptorů a jejich ligandů (např. FasR/FasL, TNFR1 [tumour necrosis factor receptor 1]/TNF-α [tumour necrosis factor α] nebo DR3 [death receptor 3]/Apo3L). Po aktivaci receptoru příslušným ligandem je proapoptotický signál přenesen dovnitř buňky na adaptorové proteiny (např. FADD [fas associated death domain] a TRADD [TNFR associated via death domain]). Tyto proteiny dále asociují s prokaspázou 8 a/nebo 10, což vede k vytvoření komplexu DISC a aktivaci prokaspáz (Kischkel et al. 1995, Martin et al. 1998, Degterev et al. 2003).

Vnitřní dráha (také označována jako mitochondriální dráha) je obecně směřována k mitochondriím, kde dochází k změnám na vnitřní mitochondriální membráně. Konkrétně k otevírání PTP (permeability transition pore) pórů, snížení mitochondriálního membránového potenciálu ($\Delta\Psi_m$) a uvolňování klíčových faktorů realizace indukce apoptózy. Tyto proapoptotické proteiny můžeme na základě jejich funkce rozdělit do dvou skupin. První skupina zahrnuje cytochrom c, Smac /DIABLO (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis-binding protein with low pI) a serinovou proteázu HtrA2 (high temperature requirement protein A2)/Omi (Du et al. 2000, Loo et al. 2002, Garrido et al. 2006). Tyto proteiny aktivují mitochondriální dráhu závislou na kaspázách. Cytochrom c se váže na protein Apaf-1 (apoptotic protease activating factor 1), který po interakci s iniciační prokaspázou 9 vytváří komplex apoptozóm (Chinnaiyan 1999, Hill et al. 2004) a aktivuje tuto kaspázu. Proapoptotická úloha proteinů Smac/DIABLO a HtrA2/Omi je dána jejich schopností inhibovat aktivitu IAP. Ty se následně nemohou vázat na kaspázy a může dojít k iniciaci apoptózy (van Loo et al. 2002, Schimmer 2004). Druhá skupina proapoptotických proteinů uvolněných z mitochondrie zahrnuje AIF (apoptosis-inducing factor), endonukleázu G a CAD (caspase-activated deoxyribonuclease). Tyto proteiny se však uplatňují v pozdější fázi apoptózy (viz níže). Regulace uvedených mitochondriálních procesů probíhá zejména prostřednictvím proteinů rodiny Bcl-2 (B-cell CLL/lymphoma-2), (Cory & Adams 2002).

Dále existuje ještě tzv. perforin/gramzym B dráha. Ta může být v buňkách spuštěna prostřednictvím cytotoxických T lymfocytů a zahrnuje účast molekul perforinu a

granzymu. Uvedené iniciační signální dráhy apoptózy mohou být vzájemně propojeny (Igney & Krammer 2002, Barry & Bleackley 2002).

5.2.2.2. Molekulární mechanizmy exekuce apoptózy

Výše popsané dráhy vedou k finální exekuční fázi apoptózy. Ta je realizována štěpením exekučních kaspáz 3, 6 a 7 iniciačními kaspázami či jinými proteázami (granzym B). Exekuční káspázy následně aktivují cytoplazmatické endonukleázy a proteázy, které degradují DNA (deoxyribonucleic acid), jaderné a cytoskeletární proteiny. Zdá se, že nejvýznamnější exekuční kaspázou je v procesu exekuce apoptózy kaspáza 3. Ta může být aktivována iniciačními kaspázami 2, 8, 9 a 10. Exekuční kaspázy štěpí řadu proteinů, které označujeme jako tzv. substráty smrti. Jedná se například o proteiny uplatňující se při štěpení DNA jako endonukleáza G a AIG, různé strukturní (kadhedriny, aktin, filamin, spektrin) a regulační (cykliny, cytokiny, transkripční faktory) proteiny (Slee et al. 2001, Fischer et al. 2003, Timmer & Salvesen 2007). Štěpení substrátů smrti vede k formování apoptotických tělísek a expozici ligandů pro receptory fagocytů. Těmi je buňka následně pohlcena, takže nedochází k zánětlivé reakci.

5.3. Vliv mastných kyselin na pankreatické β-buňky

5.3.1. Mastné kyseliny

Mastné kyseliny (MK) jsou karboxylové kyseliny s alifatickým řetězcem. U většiny přirozeně se vyskytujících MK obsahuje tento řetězec sudý počet uhlíků, většinou mezi čtyřmi a dvaceti-osmi. Z hlediska výživy a efektu MK na buňky (i pankreatické β-buňky) jsou nejvýznamnější MK s dlouhým řetězcem, které mají 14-22 uhlíků v alifatickém řetězci. V této dizertační práci je pojem „mastné kyseliny“ používán pro MK s dlouhým řetězcem (pokud není uvedeno jinak).

Podle stupně saturace můžeme MK rozdělit na nasycené, které neobsahují dvojnou vazbu ve svém alifatickém řetězci, a nenasycené MK, které obsahují jednu (mononasycené) nebo více (polynasycené) dvojných vazeb mezi atomy uhlíku. Nenasycené MK můžeme dále dělit podle isomerie (konfigurace dvojné vazby) na *cis* a *trans*, přičemž většina nenasycených MK existuje v *cis* konformaci. Míra saturace MK, stejně jako typ isomerie nenasycených MK má význam pro vlastnosti buněčných membrán, např. fluiditu (Karnovski et al. 1982).

MK mohou být vzhledem k svému hydrofobnímu charakteru transportovány do buňky prostou difúzí (Kamp & Hamilton 2006). Nicméně byly identifikovány některé proteiny, které vstup MK do buňky usnadňují či přímo zprostředkovávají (Glatz et al. 2010). Některé z nich (např. FATP [fatty acid transport protein]-1, FATP-4, FAT [fatty acid translocase]/CD36 [cluster of differentiation 36] a kaveolin-1) jsou přítomny i v pankreatických β-buňkách (Noushmehr et al. 2005, Veluthakal et al. 2005, Dalgaard et al. 2011). Vzhledem k nerozpustnosti MK ve vodě jsou experimentálně aplikovány na buňky v komplexu s bovinním sérovým albuminem (BSA).

Metabolismus MK začíná jejich konverzí na tzv. „long-chain" acylkoenzym A (LC-CoA). Následně může být tento meziprodukt v závislosti na hladině glukózy esterifikován na triacylglyceroly, či jiné deriváty jako např. ceramid. Ty mohou mít na buňky také škodlivý efekt (Poitout et al. 2010, Unger & Zhou 2001). LC-CoA může být také v mitochondriální matrix oxidován na acetylkoenzym A. LC-CoA také napomáhá fúzi granul sekretujících inzulín s plazmatickou membránou β-buněk. Může tedy rovněž přímo ovlivňovat sekreci inzulínu (Deeney et al. 2000).

MK se mohou účastnit regulace řady buněčných procesů například prostřednictvím regulace genové transkripce (Jump 2004, Georgiadi & Kersten 2012), palmitoylace a myristilace proteinů (Legrand & Rioux 2010) nebo skrze vytváření různých signálních molekul například ceramidu (Nikolova-Karakashian & Rozenova 2010, Lang et al. 2011).

5.3.2. Vliv mastných kyselin na viabilitu pankreatických β-buněk

Na začátku tohoto století bylo prokázáno, že dieta bohatá na tuky způsobuje u experimentálních zvířat úbytek pankreatických β-buněk v důsledku indukce apoptózy (Joseph et al. 2002, Sone & Kagawa 2005, Sauter et al. 2008, Hennige et al. 2010). Toto zásadní zjištění tak odstartovalo a také legitimizovalo intenzivní výzkum molekulárních mechanizmů *in vitro*, kterými je proapoptotický efekt MK zprostředkováván. Záhy se ukázalo, že efekt MK na β-buňky je závislý na délce alifatického řetězce a stupni saturace dané MK (Diakogiannaki et al. 2007, Newsholme et al. 2007, Dhayal et al. 2008). Tato problematika byla dále detailněji studována a to na zvířecích β-buněčných liniích, ale i na zvířecích a dokonce lidských izolovaných ostrůvcích. Byl zjišťován efekt zejména těch MK, které jsou nejvíce zastoupeny v krevním séru, tj. kyseliny palmitové (PA), stearové (SA), palmitolejové (POA) a olejové (OA), (Ingalls & Hoppel 1995, Lagerstedt et al. 2001).

5.3.2.1. Proapoptotický efekt nasycených mastných kyselin

Na primárních krysích β-buňkách (Cnop et al. 2007), stejně jako na krysích (Maedler et al. 2001, 2003) a lidských (Eitel et al. 2002, El Assaad et al. 2003, Maedler et al. 2003, Lai et al. 2008, Ladriere et al. 2010) ostrůvcích, bylo zdokumentováno, že nasycená PA a SA způsobují buněčnou smrt. Proapoptotický efekt nasycených MK byl také demonstrován na hlodavčích β-buněčných liniích MIN6 (Busch et al. 2005, Choi et al. 2007, Laybutt et al. 2007, Jeffrey et al. 2008, Lai et al. 2008, Thorn & Bergsten 2010), β-TC (Hirota et al. 2006), RIN1046-38 (Eitel et al. 2002, 2003), RINm5F (Baldwin et al. 2012), BRIN-BD11 (Welters et al. 2004, 2006, Diakogiannaki et al. 2008), HIT-T15 (Okuyama et al. 2003, Tuei et al. 2011, Kim et al. 2018) a INS-1 (El Assaad et al. 2003, Moffitt et al. 2005, Lin et al. 2012). Nasycené MK, které mají kratší alifatický řetězec než 16 uhlíků (např. myristát), nejsou schopné v buňkách buněčnou smrt vyvolat (Welters et al. 2004).

Proapoptotický efekt na β-buňky byl v některých studiích (Cnop et al. 2002, 2007, Maestre et al. 2003, Kharroubi et al. 2004, Karaskov et al. 2006, Cunha et al. 2008, Lai et al. 2008, Li et al. 2010, Tuo et al. 2011) demonstrován i po aplikaci nenasycených MK. Nicméně zjištěný cytotoxický efekt byl většinou slabší. Protichůdnost výsledků při sledování efektu nenasycených MK je zarážející, ale patrně plyne z odlišného nastavení pokusů v jednotlivých studiích (různá použitá koncentrace BSA a dané MK, používání séra prostého media, apod.). Navzdory určité výše uvedené nekonzistenci výsledků dnes panuje názor, že nasycené MK mají oproti nenasyceným MK výrazný proapoptotický potenciál (Diakogiannaki et al. 2007, Newsholme et al. 2007, Cnop et al. 2008, Dhayal et al. 2008, Morgan & Dhayal 2009).

5.3.2.2. Antiapoptotický efekt nenasycených mastných kyselin

Je publikováno stále více prací, které ukazují, že nenasycené MK jsou schopné inhibovat proapoptotický efekt nasycených MK. Tento efekt byl demonstrován v buněčných liniích INS-1 (Dhayal et al. 2008), INS-1E (Cunha et al. 2008), BRIN-BD11 (Welters et al. 2004, 2006, Diakogiannaki et al. 2007, Dhayal et al. 2008), RIN1046-38 (Eitel et al. 2002), a také krysích (Cnop et al. 2001, Maedler et al. 2001) a lidských ostrůvcích (Eitel et al. 2002, Maedler et al. 2003). Bylo také zjištěno, že nenasycené MK s krátkým řetězcem (s méně než šestnácti uhlíky, např. kyselina myristolejová) nemají tento inhibiční efekt nebo jsou oproti nenasyceným MK s dlouhým alifatickým řetězcem (např. POA či OA) méně účinné (Welters et al. 2004, Dhayal et al. 2008). Zdá se, že zatímco počet či umístění (pozice) dvojných vazeb v nenasycených MK nemá vliv na jejich cytoprotektivní efekt (Dhayal &

Morgan 2011), tak konfigurace dvojné vazby (typ izomerie) určitý vliv nejspíše má. V liniích BRIN-BD11 a INS-1 byl u kyseliny palmitelaidové a elaidové (*trans* protějšky POA a OA) prokázán nižší cytoprotektivní účinek oproti jejím *cis* izomerům (Dhayal et al. 2008). Dále bylo zjištěno, že nenasycené MK jsou schopny cytoprotektivního působení již při výrazně nižších koncentracích (např. 50×), než jaké jsou potřeba pro indukci apoptózy v důsledku působení nasycených MK (Welters et al. 2004, Dhayal et al. 2008). Kromě toho, nenasycené MK jsou schopny inhibovat apoptózu i pokud jsou přidány několik hodin po aplikaci nasycených MK. Dokonce jsou schopny inhibovat apoptózu vyvolanou i jinými proapoptotickými stimuly, např. cytokiny či nepřítomností séra (Welters et al. 2004, Dhayal et al. 2008, Diakogiannaki et al. 2008). Tato zjištění podporují hypotézu, že inhibiční efekt nenasycených MK na apoptózu vyvolanou nasycenými MK je zprostředkován spíše přes regulaci příslušné buněčné signalizace než skrze metabolické intervence (Welters et al. 2004, Newsholme et al. 2007, Morgan & Dhayal 2009).

5.4. Mechanizmy indukce apoptózy nasycenými mastnými kyselinami u pankreatických β-buněk

To, že mastné kyseliny způsobují buněčnou smrt β-buněk skrze indukci apoptózy, bývá dokazováno různými způsoby. Nejčastěji však prostřednictvím aktivace kaspáz nebo přítomností typického apoptického markeru fosfatidylserinu na vnějším povrchu cytoplazmatické membrány. Apoptóza může být realizována v závislosti na dané buněčné linii několika různými způsoby (dráhami).

5.4.1. Aktivace klasických drah indukce a exekuce apoptózy

V patogenezi DMT2 může hrát důležitou roli receptorová dráha apoptózy. U myší, které v β-buňkách neměly kaspázu 8 a které byly krmeny potravou bohatou na tuky, byl zjištěn menší úbytek β-buněk a nedošlo u nich k rozvoji DMT2 (Liadis et al. 2007). Kromě toho u pacientů s DMT2 byla ve srovnání s nediabetickými kontrolami zjištěna v pankreatických ostrůvcích zvýšená aktivace kaspázy 8 (Marchetti et al. 2004). Nicméně Fas receptor se velmi pravděpodobně na aktivaci kaspázy 8 a patogenezi DMT2 nepodílí. Specifická delece tohoto receptoru nebyla schopna ochránit myši proti vzniku DMT2 v důsledku diety bohaté na tuky. Ostatní receptory smrti a jejich aktivace jednotlivými MK nebyly dosud studovány.

Dále bylo zjištěno možné zapojení vnitřní (mitochondriální) dráhy apoptózy v procesu buněčné smrti indukované MK. Po aplikaci PA bylo v izolovaných krysích (Maedler et al. 2001, Choi et al. 2007) a lidských ostrůvcích (Maedler et al. 2003) a v krysích buněčných liniích (Maestre et al. 2003, Choi et al. 2007, Allagnat et al. 2011) prokázáno uvolňování cytochromu c z mitochondrií do cytoplazmy. V buňkách INS-1 byla monitorována po aplikaci PA také hladina $\Delta\Psi_m$ a ROS (reactive oxygen species), (Maestre et al. 2003). Byl zjištěn pokles $\Delta\Psi_m$ a zvýšení ROS. Vedle vlivu MK na uvolňování cytochromu c, hladinu $\Delta\Psi_m$ a ROS, byl studován vliv MK také na proteiny rodiny Bcl-2. Bylo prokázáno, že aplikace PA vede k zvýšení mRNA (messenger ribonucleic acid) nebo exprese proapoptotických členů rodiny Bcl-2 (např. Bax [Bcl-2-associated X protein]), (Maestre et al. 2003, Wang et al. 2010). Hladina antiapoptotických členů rodiny Bcl-2 (např. Bcl-2) se nezměnila (Maestre et al. 2003) nebo poklesla (Maedler et al. 2003, Allagnat et al. 2011). Nenasycené POA a OA měly oproti PA opačný efekt (Maedler et al. 2003). Zvýšená hladina proteinu Bax byla pozorována i v pankreatických β-buňkách pacientů s DMT2 (Laybutt et al. 2007).

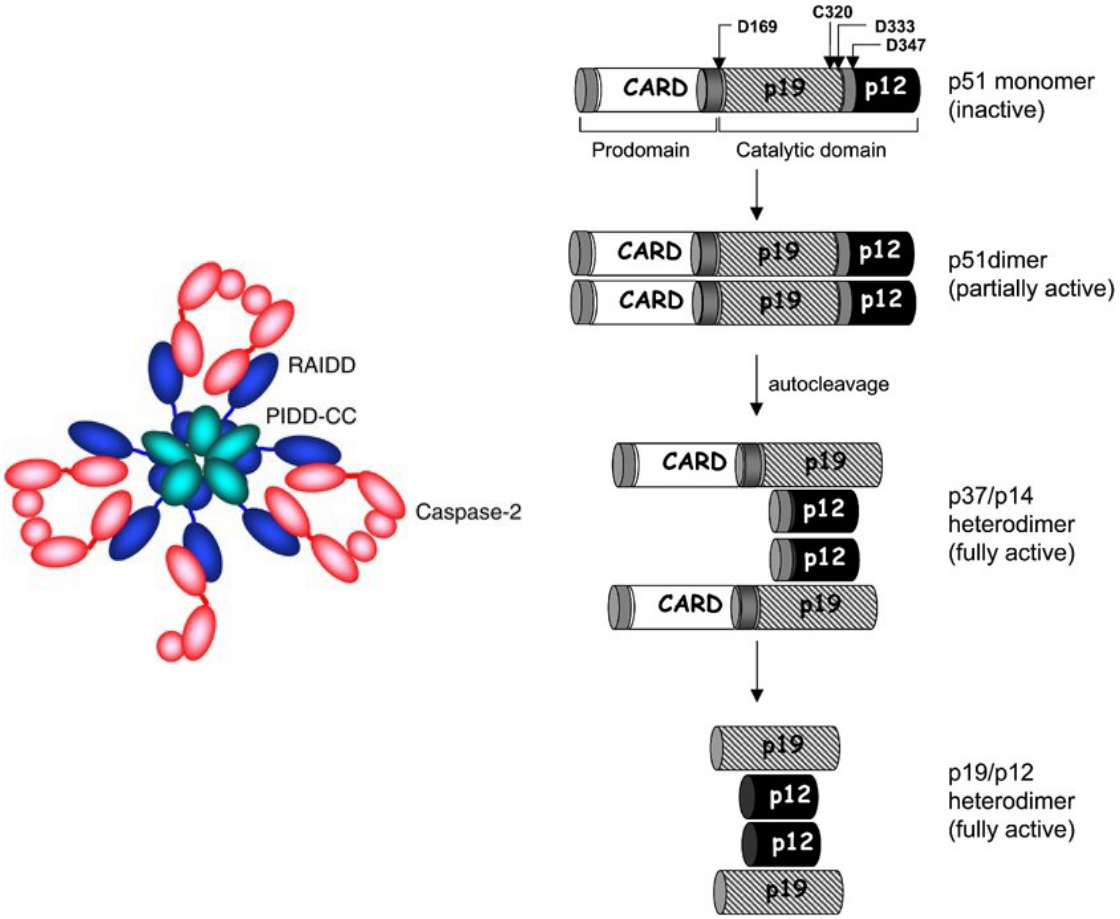
Vliv proapototických MK na štěpení exekučních kaspáz byl dosud nejvíce studován na kaspáze 3. Aktivace této kaspázy byla prokázána po aplikaci PA a/nebo SA v MIN6 (Johnson et al. 2004, Choi et al. 2007, Thorn et al. 2010, Wang et al. 2010), INS-1 (Rakatzi et al. 2004, Choi et al. 2007), INS-1E (Bachar et al. 2009, Allagnat et al. 2011), INS 832/13 (El Assaad et al. 2003), RIN1046-38 (Eitel et al. 2003) and β-TC1 (Hirota et al. 2006) buněčných liniích a také v izolovaných krysích (Choi et al. 2007) a lidských ostrůvcích (Maedler et al. 2003). V buněčné linii BRIN-BD11 (Dhayal et al. 2008) a primárních krysích β-buňkách (Cunha et al. 2008) byla aktivace této kaspázy také prokázána, ale hladina zjištěné aktivace zahrnovala i aktivaci kaspázy 7 (díky nespecifičnosti použité metody). Dále byla po aplikaci PA prokázána aktivace kaspázy 6. Její aktivace předcházela aktivaci kaspázy 3 (Hirota et al. 2006). Vedle sledovaní efektu MK na samotnou aktivaci kaspáz se dále zjišťovalo, do jaké míry kaspázy zprostředkovávají apoptózu indukovanou MK. Pomocí aplikace obecného inhibitoru kaspáz z-VAD-fmk společně s MK bylo prokázáno (Lupi et al. 2002, Welters et al. 2006), že kaspázy mají při indukci apoptózy MK významnou úlohu, ale že se tohoto procesu účastní ještě další proteázy.

5.4.2. Kaspáza 2 a indukce apoptózy

Kaspáza 2 představuje evolučně nejvíce konzervovanou savčí kaspázu. Jak již bylo zmíněno výše, kaspáza 2 reprezentuje iniciační kaspázu a pravděpodobně funkčně nejvíce apikální kaspázu (Lassus et al. 2002, Enoksson et al. 2004). Za normálních podmínek se kaspáza 2 v buňce vyskytuje ve formě inaktivních prekurzorů – prokaspázy 2. Ta se skládá z velké a malé podjednotky, které představují katalytickou doménu, a tzv. prodomény, která plní funkci inhibiční podjednotky proteinu.

K aktivaci kaspázy 2 dochází v komplexu označovaném jako PIDDozóm. Ten se skládá z kaspázy 2, proteinů PIDD (p53-induced protein with a death domain) a RAIDD (RIP-associated protein with a death domain), (Tinel & Tschopp 2004). Nejprve pravděpodobně dochází v důsledku příslušného proapoptotické signálu k interakci proteinu RAIDD, přes jeho „death" doménu, s proteinem PIDD. Následně se prokaspáza 2, která se v buňkách vyskytuje ve formě monomerů, váže prostřednictvím své CARD (caspase recruitment domain) domény na adaptorový protein RAIDD (viz obr. 1, str. 24). V důsledku toho dochází k přiblížení molekul prokaspázy 2. Při dostatečném přiblížení těchto molekul dojde k interakci proteázových domén kaspázy 2, které vytváří dočasný dimerický komplex, který již má určitou katalytickou aktivitu. Dimerizace je následována štěpením v místě mezi malou a velkou podjednotkou proteázových domén jednotlivých monomerů. To vede k formování aktivního stabilního tetramerického komplexu, který již má maximální katalytickou aktivitu. Následné štěpení (autokatalytické nebo jinou kaspázou) vede k vytvoření finálního enzymu kaspázy 2 (viz obr. 1, str. 24). Aktivace kaspázy 2 nezávislá na proteinech RAIDD a PIDD byla také dokumentována (Mansilla et al. 2006, Manzl et al. 2009).

Dosavadní poznatky týkající se úlohy kaspázy 2 v mechanizmech indukce apoptózy jsou značné. Řada těchto poznatků je však rozporuplných. Proto je obtížné přesně definovat úlohu této kaspázy v rámci procesu indukce apoptózy (Bouchier-Hayes & Green 2012). Byla prokázána aktivace kaspázy 2 v důsledku poškození DNA. Dále se zdá, že tato kaspáza má určitou úlohu v rámci zablokování buněčného cyklu a buněčné smrti vyvolané tzv. mitotickou katastrofou (Mansilla et al. 2006, Manzl et al. 2009). Poměrně nově byla také prokázána možná úloha této molekuly v rámci apoptózy vyvolané stresem endoplazmatického retikula (ER), (Cheung et al. 2006, Upton et al. 2008). Co se týče potenciálního zapojení kaspázy 2 v rámci mechanizmů indukce apoptózy nasycenými MK byla publikována zatím pouze jediná práce, kde však autoři nesledovali aktivaci této kaspázy (Maestre et al. 2003).



Obrázek 1. Navrhovaný model PIDDozómu (vlevo, převzato z Bouchier-Hayes & Green 2012) a mechanizmus aktivace kaspázy 2 (vpravo, převzato z Baliga et al. 2004). Vysvětlení viz kapitola 5.4.2., str. 23.

5.4.3. p38 MAPK signální dráha a indukce apoptózy

V rámci molekulárních mechanizmů indukce apoptózy MK u pankreatických β-buněk může být zapojena mimo jiné i p38 MAPK (mitogen-activated protein kinase) signální dráha. Klíčovou molekulu představuje v rámci této dráhy serin-treonin kináza p38 MAPK. Dosud byly identifikovány čtyři izoformy p38 MAPK: p38α, p38β (Jiang et al. 1996), p38γ (Lechner et al. 1996, Li et al. 1996) a p38δ (Jiang et al. 1997, Kumar et al. 1997). Tyto izoformy sdílí přibližně 60 % sekvence DNA a bývají klasifikovány podle Thr-Gly-Tyr (TEY) duálního fosforylačního motivu. p38 MAPK je aktivována fosforylací threoninu a tyrosinu prostřednictvím kinázy MKK (mitogen-activated protein kinase kinase) 3/6 nebo v některých typech buněk MKK4 kinázou (Cohen 1999, Kyriakis et al. 2001). Dále je zde ještě nejméně jeden mechanizmus aktivace p38 MAPK nezávislý na MKK a zahrnující protein TAB1 (transforming growth factor-β-activated protein kinase 1 (TAK1)-binding protein), (Matsuyama et al. 2003, Tanno et al. 2003). Jako další „upstream“ aktivátory byly

identifikovány různé MAP3Ks (mitogen-activated protein kinase kinase kinase) jako např. TAK1 (transforming growth factor-β-activated protein kinase 1), (Moriguchi et al. 1996), ASK1 (apoptosis signal-regulating kinase 1), (Ichijo et al. 1997), MLKs (mixed-lineage kinases), (Zhang et al. 1995), a některé další proteiny rodiny MEKK (Cuenda & Dorow 1998, Takekawa et al. 1997). K aktivaci p38 MAPK „upstream" od MAP3Ks mohou přispívat některé GTP (guanosin trifosfát)-vazebné proteiny podrodiny Rho jako např. Rac1 (Ras-related C3 botulinum toxin substrate 1), Cdc42 (cell division control protein 42) nebo Rho (Zhang et al. 1995, Bagrodia et al. 1995). Ty jsou aktivovány v důsledku různých extracelulárních fyzikálních (UV, teplo) a chemických (anisomycin) stimulů a cytokinů (tumor necrosis factor α [TNF-α], colony stimulating factor 1 [CSF-1]), (přehledně v Zarubin & Han 2005). p38 MAPK může v závislosti na buněčném typu regulovat aktivaci různých proteinů, např. transkripčních faktorů (CHOP [CCAAT-enhancer-binding protein C/EBP], ATF2 [activating transcription factor 2]), jiných protein kináz (MAPKAPK-2 [MAP kinase-activated protein kinase 2] a 3, MSK1/2 [mitogen- and stress-activated protein kinase 1 and 2]), komponentů translační mašinérie (eIF-4E [eukaryotic translation initiation factor 4E]) a dalších (přehledně v Cuenda & Rousseau 2007). Tato signální dráha se může podílet, v závislosti na buněčném typu, na regulaci různých procesů jako je buněčný cyklus, diferenciace, senescence, autofagie, proces zánětlivé reakce, a i na regulaci indukce apoptózy (Zarubin & Han 2005, Liu et al. 2015).

Co se týče funkce p38 MAPK signální dráhy v pankreatických β-buňkách, byla zjištěna úloha této dráhy (zejména kinázy p38 MAPK) při diferenciaci (linie AR42J; Kojima & Umezawa 2006), proliferaci (primární krysí β-buňky; Parnaud et al. 2009) nebo senescenci (Langerhansovy ostrůvky C57BL/6J myší, Sone & Kagawa 2005) těchto buněk. Nejčastěji je však tato dráha studována v souvislosti s funkcí a zejména viabilitou β-buněk ovlivněnou různými faktory, včetně MK (např. Saldeen & Welsh 2004, Yang et al. 2007, Lu et al. 2011). Na pankreatických β-buněčných liniích či ostrůvcích bylo prokázáno, že p38 MAPK může být po aplikaci MK aktivována, případně přímo zprostředkovávat proapoptotickou signalizaci indukovanou působením MK (Zhang et al. 2007a, Cvjetićanin et al. 2009, Natalicchio et al. 2013).

5.4.4. ERK signální dráha a indukce apoptózy

Také ERK signální dráha se může podílet na mechanizmech indukce apoptózy MK u pankreatických β-buněk. Zřejmě nejvýznamnější kinázu představuje v rámci této dráhy ERK1/2 (extracellular signal-regulated kinase 1/2, také známá jako p42/p44 MAPK). ERK

dráha bývá v závislosti na buněčném typu aktivována různými stimuly, zejména růstovými faktory a to přes příslušné RTKs (receptor tyrosine kinase). Ty, prostřednictvím adaptorového proteinu SOS (son of sevenless) aktivují Ras (rat sarcoma) GTPázu, která zapojuje c-Raf kinázu do komplexu, kde proběhne její aktivace (Chang et al. 2003). c-Raf fosforyluje dvě serinová rezidua na MEK1/2 (mitogen-activated protein kinase/ERK kinase) kináze. Tato kináza následně aktivuje ERK1/2. c-Raf může být kromě proteinu Ras aktivována také jinými proteiny jako např. PKC (protein kinase C), (Kolch et al. 1993), Src (Fabian et al. 1993), PAK (p21-activated protein kinase), (Diaz et al. 1997) a dalšími. Nicméně byly zjištěny i molekuly, které c-Raf inhibují, jako například Akt (Reusch et al. 2001), PKA (protein kinase A), (Dhillon et al. 2002a), PP2a (protein phosphatase 2a), (Dhillon et al. 2002b) nebo právě výše uvedená p38 MAPK (Lee et al. 2002). Zdá se, že ihned po fosforylaci ERK1/2 dochází k její translokaci do jádra (Pouysségur et al. 2002). Tato kináza může následně regulovat aktivaci řady substrátů v různých buněčných kompartmentech (přehledně v Chen et al. 2001) jako například různých kináz (RSKs [p90 ribosomal S6 kinases], MSKs, MNKs [MAP kinase signal-integrating kinases]), cytoskeletálních proteinů (paladin a paxillin), transkripčních faktorů (c-Fos, c-Myc) nebo membránových proteinů (Syk [spleen tyrosine kinase], calnexin). ERK signální dráha se obecně uplatňuje jako klíčový regulátor proliferace. Může se však v některých typech buněk podílet i na regulaci diferenciace, autofagie, senescence a apoptózy (přehledně v Cagnol & Chambard 2009).

ERK signální dráha dosud nebyla v pankreatických β-buňkách komplexně studována. Bylo zjištěno, že tato dráha zprostředkovává signalizaci vyvolanou inzulínem (Langerhansovy ostrůvky myší kmenu C57BL6/J; Alejandro et al. 2010). Dále byla prokázána její úloha při proliferaci (linie INS-1; Kim et al. 2014) β-buněk. Zejména pak byla zjišťována její funkce ve vztahu k viabilitě β-buněk ovlivněné různými faktory i MK (např. Kang et al. 2007, Simon et al. 2008, Wijesekara et al. 2010, Youl et al. 2010). Vliv MK na aktivaci ERK1/2 byl dokumentován v MIN6, INS-1, RINm5F, NIT-1 buněčných liniích (Zhang et al. 2007a, Simon et al. 2008, Fontés et al. 2009, Plaisance et al. 2009, Guo et al. 2010, Watson et al. 2011). Nicméně ze zjištěných výsledků není jasné, zda tato dráha přenáší pro- či antiapoptotickou signalizaci. Efekt jednotlivých, jak nasycených tak nenasycených MK na aktivaci ERK1/2 totiž není v jednotlivých studovaných liniích koncizní.

5.4.5. Stres endoplazmatického retikula (ER) a indukce apoptózy

Narušení funkce ER může vést k indukci buněčné smrti. Tato narušení se obecně označují jako stres ER. Pokud k nim dojde, dochází v buňce k aktivaci ochranných mechanizmů označovaných jako „ER stress response“ nebo „unfolded protein response“ (UPR), (Woehlbier & Hetz 2011). Pankreatické β-buňky jsou, vzhledem k jejich specializaci na sekreci inzulínu, velmi citlivé na jakékoliv narušení funkce ER. Patří tedy mezi buňky nejvíce náchylné k stresu ER (Araki et al. 2003).

Na UPR signalizaci se u buněk obratlovců zásadním způsobem podílí tři proteiny – IRE1α (inositol-requiring protein 1α), PERK (protein kinase RNA-like endoplasmic reticulum kinase) a ATF6 (activating transcription factor 6). Jsou lokalizovány v membráně ER a přenášejí informaci o situaci v ER do cytosolu a jádra, v důsledku čehož dojde k nápravě správné funkce ER. Za normálního stavu jsou tyto proteiny inaktivní pravděpodobně díky interakci s hlavním chaperonem ER, proteinem BiP (immunoglobulin binding protein, také znám jako glucose-regulated protein, Grp78 [glucose-regulated protein 78]), (Hetz et al. 2011). Při stresu ER dochází k disociaci proteinu BiP od těchto tří proteinů.

IRE1α následně dimerizuje, což vede k autofosforylaci a aktivaci cytosolické endoribonukleázy a kinázové domény (Woehlbier & Hetz 2011). Aktivované IRE1α realizuje alternativní splicing mRNA proteinu XBP1 (X-box binding protein 1), což následně vede k produkci aktivního a stabilního transkripčního faktoru XBP1s. Tento protein je translokován do jádra. Zde zvýšuje expresi genů kódujících proteiny, které se podílí na degradaci špatně sbalených proteinů v lumen ER (tzv. „ER-associated degradation“ [ERAD]), na regulaci vstupu proteinů do ER, na formování proteinů do správné konformace a dalších funkcích (Acosta-Alvear et al. 2007). Aktivní IRE1α také inter-reaguje s proteinem TRAF2 (TNFR-associated factor 2), což vede k aktivaci JNK (c-Jun N-terminal kinase), (Urano et al. 2000). JNK přenáší proapototickou signalizaci „downstream“ od signálních drah stresu ER (Woehlbier & Hetz 2011).

PERK je aktivován po disociaci proteinu BiP autofosforylací a homomultimerizací. Aktivovaný PERK fosforyluje α podjednotku translačního faktoru eIF2 (eukaryotic initiation factor 2α). To vede k obecnému snížení translace. Nicméně, translace některých mRNA je touto fosforylací naopak selektivně zvýšena, např. mRNA kódující ATF4 (activating transcription factor 4). Ten reguluje hladinu různých prosurvival genů ovlivňujících řádné sbalování proteinů, autofagii nebo metabolizmus aminokyselin (Woehlbier & Hetz 2011). ATF4 také výrazně zvyšuje expresi proteinu CHOP, který je

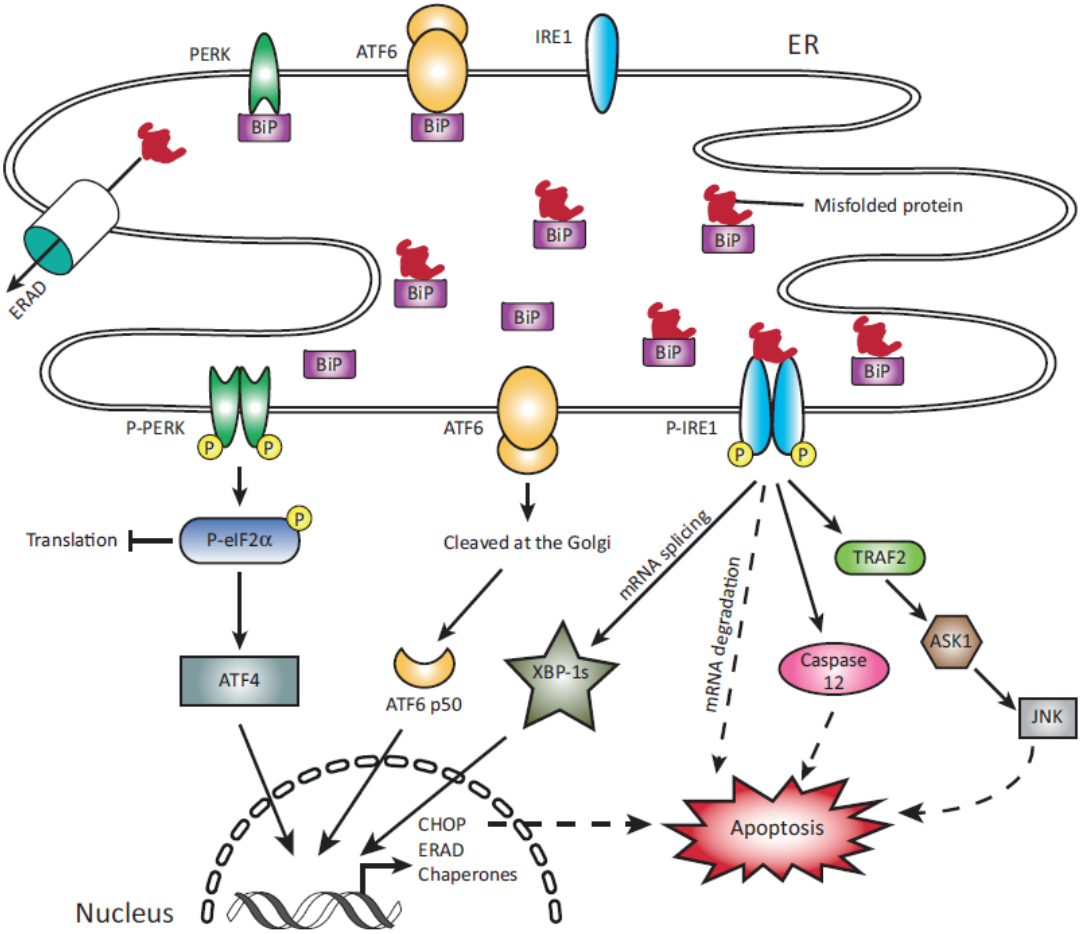
pravděpodobně zapojený do procesu indukce apoptózy stresem ER (Nishitoh 2012). Důležitým transkripčním cílem ATF4 a CHOP je GADD34 (growth arrest and DNA damage-inducible protein 34), který umožňuje defosforylaci fosforylovaného eIF2α, což vede k obnovení intenzity obecné translace a snížení translace ATF4 na bazální úroveň (Ma & Hendershot 2003).

ATF6 je po disociaci BiP translokován do Golgiho aparátu, kde je štěpen proteázami (Haze et al. 1999). To vede k uvolnění cytosolické části ATF6 označované jako ATF6f. Ta pak funguje jako transkripční faktor a přímo reguluje expresi genů kódujících chaperony, komponenty ERAD a XBP1 (Woehlbier & Hetz 2011).

Prvotní odpovědí buňky při stresu ER je tedy dočasná inhibice obecné syntézy proteinů. To buňce poskytuje čas, kdy se může vymanit ze stresového působení a navrátit se do homeostázy prostřednictvím aktivace dalších drah UPR popsaných výše. Pokud však stresový stimul přetrvává, stane se tento obranný mechanizmus buňky neúčinný a dojde k indukci apoptózy (Araki et al. 2003). Mechanizmy indukce apoptózy „downstream“ od stresu ER jsou dosud nejasné. Předpokládá se, však zapojení transkripčního faktoru CHOP, proteinů rodiny Bcl-2, JNK kinázy, aktivace kaspázy 12 a možná i kaspáz 2 a 4 (Rodriguez et al. 2011, Tabas & Ron 2011). Zjednodušené schéma dosavadních poznatků týkajících se drah stresu ER, mechanizmů UPR a apoptózy vyvolané stresem ER je na obrázku 2. (str. 29).

Zapojení stresu ER v patogenezi DMT2 bylo prokázáno v řadě studií provedených jak na zvířecích, tak lidských buňkách (Thornton et al. 1997, Oyadomari et al. 2002, Fonseca et al. 2010), ale i na Langerhansových ostrůvcích pacientů s DMT2 (Huanga et al. 2007a, Laybutta et al. 2007, Marchetti et al. 2007). Dostupné výsledky efektu MK na aktivaci drah stresu ER v pankreatických β-buňkách jsou následující. Co se týče IRE1α dráhy, výsledky nejsou úplně jednoznačné. Aktivace této dráhy byla však většinou prokázána jak v různých hlodavčích β-buněčných liniích (Kharroubi et al. 2004, Karaskov et al. 2006, Cnop et al. 2007, Laybutt et al. 2007, Gwiazda et al. 2007, Cunha et al. 2008, Bachar et al. 2009), tak v krysích primárních β-buňkách a v lidských ostrůvcích (Cunha et al. 2008) po aplikaci PA. Aplikace nenasycených MK pak vedla k aktivaci této dráhy pouze asi v polovině prací, kde se touto problematikou zabývali (Kharroubi et al. 2004, Karaskov et al. 2006, Cnop et al. 2007, Laybutt et al. 2007, Cunha et al. 2008). Aktivace PERK dráhy (PERK-eIF2α-ATF4-CHOP) byla většinou pozorována po působení PA, a to v hlodavčích liniích, krysích primárních β-buňkách a lidských ostrůvcích. Nenasycené MK pak tuto dráhu spíše neaktivovaly (Karaskov et al. 2006, Cnop et al. 2007, Laybutt et al.

2007, Cunha et al. 2008, 2009, Diakogiannaki et al. 2008, Gwiazda et al. 2009, Ladriere et al. 2010, Thorn et al. 2010, Wang et al. 2010). Experimentální data týkající se aktivace ATF6 MK jsou nejednoznačná. Aktivace této dráhy byla zjištěna v MIN6 buňkách po aplikaci PA, ale ne OA (Laybutt et al. 2007). V linii INS-1 naopak OA také aktivovala tuto dráhu (Kharroubi et al. 2004).



Obrázek 2. Přehled klasických drah stresu endoplazmatického retikula (ER), tj. ATF6, IRE1α a PERK dráhy, které se uplatňují při obnovení funkce ER a přežívání buňky. Dále adaptivní odpovědi „downstream" od aktivace těchto drah a vybrané molekuly, které jsou považovány za případný spojující článek mezi stresem ER a indukcí apoptózy. Přerušované šipky ilustrují přechodový krok z adaptivní odpovědi k apoptóze. Převzato z Biden et al. (2014).

Zvýšená exprese chaperonu BiP je typický marker stresu ER. Nicméně, efekt MK na expresi tohoto proteinu se ukazuje být v β-buňkách značně variabilní. V některých studiích na pankreatických β-buněčných liniích nebyla po aplikaci různých MK pozorována zvýšená exprese BiP (Karaskov et al. 2006, Diakogiannaki et al. 2008, Lai et al. 2008). V některých pracích naopak ano, a sice po aplikaci PA a OA (Kharroubi et al. 2004, Cnop et al. 2007). Stejný efekt byl také zdokumentován v krysích (Ladriere et al. 2010) a lidských (Cunha et al. 2008) ostrůvcích a v MIN6 buňkách (Laybutt et al. 2007) po

aplikaci PA, ale ne OA. Zvýšená exprese proteinu BiP také představuje důležitý antiapoptotický mechanizmus. Jeho zvýšená exprese se ukázala být v MIN6 buňkách (Laybutt et al. 2007), ne ale v linii INS-1 (Lai et al. 2008), protektivní při apoptóze vyvolané PA. Z výše uvedených výsledků vyplývá, že úloha proteinu BiP v apoptóze pankreatických β-buněk vyvolané MK není dosud objasněna.

Dosavadních výsledků týkajících se mechanizmů jakým stres ER vyvolaný MK spouští apoptózu v β-buňkách je velmi málo. Jako potenciální mediátory této signalizace se ukazují být molekuly CHOP, JNK kináza a kaspáza 12 (Cunha et al. 2008).

5.4.6. Další mechanizmy indukce apoptózy

Zvýšená zásoba nasycených MK v β-buňkách může vést k *de novo* produkci ceramidu (Véret et al. 2011). Výsledky řady prací provedených na krysích a lidských buněčných liniích a ostrůvcích jasně naznačují, že se ceramid podílí na indukci apoptózy po aplikaci MK (Shimabukuro et al. 1998, Maedler et al. 2001, 2003, Guo et al. 2010). Dále byla zjišťována možná úloha oxidu dusnatého (NO). V pankreatických β-buňkách má nízká koncentrace NO produkovaného konstitutivní NO syntázou (NOS) funkci fyziologického regulátoru sekrece inzulínu (Kaneko et al. 2003) a antiapoptotický účinek (Noguchi et al. 2008). Oproti tomu zvýšená koncentrace NO v β-buňkách produkovaného inducibilní NO syntázou (iNOS) vede k apoptóze pankreatických β-buněk (Kaneto et al. 1995, Darville & Eizirik 1998, Kutlu et al. 2003). Indukce iNOS mRNA a/nebo produkce NO byla prokázána v ostrůvcích ZDF (zucker diabetic fatty) krys (Shimabukuro et al. 1998) a v buňkách linie HIT-T15 po aplikaci PA (Okuyama et al. 2003). Nicméně, v některých pracích se v krysích (Cnop et al. 2001) a lidských (Lupi et al. 2002) ostrůvcích a BRIN-BD11 buňkách (Welters et al. 2004) tento efekt po aplikaci PA nepodařilo prokázat. Dále byla zjištěna možná úloha řady dalších mechanizmů či signálních drah, například PKCδ (Eitel et al. 2003, Simon et al. 2008), PKB (protein kinase B), (Higa et al. 2006, Fontés et al. 2009), calpainu 10 (Johnson et al. 2004) nebo NF-κB (nuclear factor kappa B), (Kharroubi et al. 2004).

5.5. Vliv hypoxie na pankreatické β-buňky

5.5.1. Hypoxie

Hypoxie je jev, při kterém dochází v tkáni k poklesu hladiny pO_2 (partial pressure of oxygen) pod normální úroveň. To nastává, když spotřeba kyslíku převyšuje jeho příjem. Koncentrace kyslíku (pO_2) v arteriální krvi je přibližně 13 %, nicméně v jednotlivých

tkáních/orgánech bývá výrazně nižší a pohybuje se v rozmezí přibližně od 1 % v kůži po 10 % v ledvinách. V jednotlivých buňkách je pak koncentrace kyslíku v rozmezí 1,3-2,5 % (přehledně v Carreau et al. 2011). Hypoxie vede k metabolické krizi projevující se poklesem produkce ATP a zvýšenou produkcí ROS, čímž negativně působí na viabilitu postižených buněk a tkání. Pokud buňka trpí nedostatkem kyslíku, dojde v ní k aktivaci specifické adaptivní odpovědi zahrnující aktivaci transkripčních faktorů rodiny HIF (hypoxia-inducible factor). Ty zprostředkovávají přechod z aerobní mitochondriální produkce na anaerobní glykolytickou produkci ATP (přehledně v Cantley et al. 2010). Zároveň v postižené buňce dochází k procesům zabraňujícím vzniku ROS, jako je inaktivace pyruvát-dehydrogenázy nebo zvýšení mitochondriální autofagie (Semenza 2010, Taylor 2008).

5.5.2. Vliv hypoxie na viabilitu pankreatických β-buněk

Pankreatické β-buňky jsou citlivé k hypoxickému stresu. Jejich sekreční funkce totiž silně závisí na stimulaci mitochondriálního metabolismu, který spotřebovává velké množství kyslíku. Stanovit fyziologickou koncentraci kyslíku v pankreatu, případně v Langerhansových ostrůvcích, je technicky velmi problematické. Nicméně bylo zjištěno, že se fyziologická koncentrace kyslíku na okraji krysích pankreatických ostrůvků pohybuje v rozmezí 6,3-6,4 % (Carlsson et al. 1998). To bylo potvrzeno i *in silico* (Buchwald et al. 2011). Hypoxie bývá v pankreatu často způsobena tzv. syndromem spánkové apnoe. Ten je diagnostikován u 30-40 % obézních lidí (Valencia-Flores et al. 2000) a vyvolává hypoxii v různých tkáních. Přímý proapoptotický efekt hypoxie na především hlodavčí pankreatické β-buňky *in vivo* a i *in vitro* byl jasně prokázán (Lai et al. 2009, Pedraza et al. 2012, Zheng et al. 2012, Fang et al. 2014). Úlohu hypoxií indukované apoptózy β-buněk v patogenezi DMT2 podporuje i detekce hypoxie *in vivo* v ostrůvcích zvířecích modelů pro DMT2 (Jonas et al. 1999, Li et al. 2006, Sato et al. 2011, Bensellam et al. 2012).

5.5.3. Mechanizmy indukce a exekuce apoptózy hypoxií u pankreatických β-buněk

Molekulární mechanizmy indukce a exekuce apoptózy v důsledku hypoxie nejsou u pankreatických β-buněk příliš známy. Zdá se však, že jednu z hlavních úloh má transkripční faktor HIF-1. Určitou roli však sehrávají i další proteiny.

Bylo zjištěno, že spotřeba kyslíku stimulovaná glukózou, způsobuje v β-buňkách vnitrobuněčnou hypoxii. To vede v hlodavčích a lidských Langerhansových ostrůvcích k aktivaci transkripčních faktorů rodiny HIF (HIF-1α a HIF-2α) a následné transkripci cílových genů (přehledně v Bensellam et al. 2012). Mezi cílové geny transkripčního faktoru HIF-1 patří řada genů regulujících energetický metabolismus, dále angiogenezi, buněčnou diferenciaci a apoptózu (přehledně v Liu et al. 2012). HIF-1α a HIF-2α jsou transkripční faktory, které jsou za normální koncentrace kyslíku přítomny v cytosolu. Jsou hydroxylovány na prolinových zbytcích hydroxylázami regulovanými kyslíkem (Semenza et al. 2004). To vede k jejich rozpoznání a ubiquitinaci von Hippel-Lindau (VHL) E3 ubiquitin ligázou (Maxwell et al. 1999). V hypoxických podmínkách však nejsou HIF-1α a HIF-2α proteiny dále degradovány. To jim umožňuje dimerizovat s HIF-1β, vytvořit tak transkripční faktor HIF-1 a transportovat se do jádra (Jiang et al. 1996, Liu et al. 2012). HIF-1 prostřednictvím zvýšení exprese GLUT1 (glucose transporter 1), několika glykolytických enzymů, LDH (lactat dehydrogenase), (Semenza et al. 1996) a PDK1 (phosphoinositide-dependent protein kinase 1), (Kim et al. 2006) přenastaví v β-buňkách spotřebovávání glukózy aerobním metabolismem na spotřebovávání anaerobním metabolismem. Glykolýza vede k udržení dostatečné hladiny ATP za současného snížení spotřeby kyslíku v mitochondriích (Papandreou et al. 2006) a reguluje sekreci inzulínu (přehledně v Gerber & Rutter 2016). Zdá se, že v pankreatických β-buňkách zprostředkovává HIF-1 adaptivní odpověď na sníženou hladinu kyslíku (Stokes et al. 2013). Jeho aktivita je zřejmě nezbytná pro správné fungovaní těchto buněk (Gunton et al. 2005, Cheng et al. 2010). Některé práce však naznačují možnou úlohu tohoto komplexu při narušení funkce β-buněk (Cantley et al. 2009, Puri et al. 2009, Zehetner et al. 2008) a v indukci apoptózy v důsledku hypoxie (Moritz et al. 2002). Komplex HIF-1 má tedy v β-buňkách pravděpodobně prosurvival funkci, pouze pokud je jeho aktivita udržována na nízké hladině. Narušení jeho funkce stejně jako jeho déle trvající aktivita vede pak k narušení funkce β-buněk.

Co se týče možného zapojení receptorové dráhy indukce apoptózy v rámci molekulárních mechanizmů aktivovaných v důsledku hypoxie v pankreatických β-buňkách v jedné práci byla popsána aktivace kaspázy 8 (Lai et al. 2009). V další práci však nebylo prokázáno zapojení Fas a TRAIL (TNF-related apoptosis-inducing ligand) receptorů a proteinu c-FLIP (cellular FLICE-like inhibitory protein), (Wang et al. 2012). Jiné molekuly této dráhy nebyly zatím testovány. Co se týče možného zapojení vnitřní (mitochondriální) dráhy indukce apoptózy v důsledku hypoxie, dostupná data naznačují potenciální úlohu

některých proteinů rodiny Bcl-2. Konkrétně BNIP3 (Bcl-2/adenovirus E1B 19kDa interacting protein 3), Bcl-2, Bcl-xl (B-cell leukemia/lymphoma-extra large), Bid (BH3-interacting domain) a Bax (Moritz et al. 2002, Miao et al. 2006, Lai et al. 2009, Wang et al. 2012, Fang et al. 2014). Zapojení proteinu Bad (Bcl-2-associated death promoter) pak prokázáno nebylo (Lai et al. 2009). Efekt hypoxie na aktivaci exekučních kaspáz byl dosud studován téměř výhradně na kaspáze 3 (Ryu et al. 2009, Zheng et al. 2012, Wang et al. 2012, Tian et al. 2013, Sato et al. 2014, Qiao et al. 2015, Bensellam et al. 2016). Pedraza et al. (2012) naznačili také možnou úlohu kaspázy 7.

Bylo zjištěno, že v rámci molekulárních mechanizmů indukce apoptózy v důsledku hypoxie v pankreatických β-buňkách se může uplatňovat také signalizace stresu ER. Nicméně její úloha není vzhledem k protichůdným výsledkům (Zheng et al. 2012, Bensellam et al. 2016) v tomto procesu jasná. Zheng et al. (2012) prokázali aktivaci UPR a zvýšení exprese proapoptotického chaperonu CHOP v důsledku hypoxie, zatímco Bensellam et al. (2016) zjistili, že hypoxie inhibuje UPR. Zvýšenou expresi proteinu CHOP v důsledku hypoxie detekovali i Sato et al. (2014). I autofagie se může určitým způsobem uplatňovat v rámci molekulárních mechanizmů indukce apoptózy v důsledku hypoxie, ačkoliv její konkrétní úloha není příliš jasná. Mohla by představovat protektivní adaptivní mechanizmus, který pro hladovějící buňku zprostředkovává energii (Wang et al. 2012). Vedle výše zmíněných proteinů se může podílet na molekulárních mechanizmech indukce apoptózy v důsledku hypoxie také NF-κB dráha (Lai et al. 2009). Dále bylo zjištěno zapojení např. AMPK (adenosine monophosphate-activated protein kinase), (Ryu et al. 2009), JNK a iNOS (Ko et al. 2008).

6. KOMENTÁŘ K PREZENTOVANÝM PUBLIKACÍM

6.1. Přehled publikací

PUBLIKACE 1

Němcová-Fürstová V, **Šrámek J**, Balušíková K, James RF, Kovář J:
Caspase-2 and JNK activated by saturated fatty acids are not involved in apoptosis induction but modulate ER stress in human pancreatic β-cells.
Cellular Physiology and Biochemistry, 31: 277–289, 2013. **IF: 3.550** (2013)

PUBLIKACE 2

Šrámek J, Němcová-Fürstová V, Balušíková K, Daniel P, Jelínek M, James RF, Kovář J:
p38 MAPK is activated but does not play a key role during apoptosis induction by saturated fatty acid in human pancreatic β-cells.
International Journal of Molecular Sciences, 17: 159, 2016. **IF: 3.226** (2016)

PUBLIKACE 3

Šrámek J, Němcová-Fürstová V, Kovář J:
Kinase signaling in apoptosis induced by saturated fatty acids in pancreatic β-cells.
International Journal of Molecular Sciences, 17: 1400, 2016. **IF: 3.226** (2016)

PUBLIKACE 4

Šrámek J, Němcová-Fürstová V, Kovář J:
Effect of saturated stearic acid on MAP kinase and ER stress signaling pathways during apoptosis induction in human pancreatic β-cells is inhibited by unsaturated oleic acid.
International Journal of Molecular Sciences, 18: 2313, 2017. **IF: 3.226** (2016)

PUBLIKACE 5

Šrámek J, Němcová-Fürstová V, Kovář J:
Hypoxia modulates effects of fatty acids on human pancreatic β-cells.
International Journal of Molecular Sciences, připraveno k odeslání, 2018.

6.2. Vlastní komentář k publikacím a diskuze výsledků

Hledání efektivní léčby diabetu mellitu 2. typu (DMT2) dnes představuje, vedle snahy nálezt efektivnější léčbu nádorových a kardiovaskulárních onemocnění celosvětově, jednu z hlavních priorit medicíny. Je tomu tak zejména kvůli velkému a stále se zvyšujícímu počtu pacientů a také kvůli vzrůstajícím nákladům na léčbu. Z těchto důvodů jsou znalosti mechanizmů patogeneze této choroby velmi potřebné, neboť mohou pomoci při hledání nových možností diagnózy, léčby a prevence. Výzkum v naší laboratoři je zaměřen na studium molekulárních mechanizmů, které se podílejí na dysfunkci a apoptóze pankreatických β-buněk ve spojitosti s patogenezí DMT2. Konkrétně se snažíme přispět k pochopení molekulárních mechanizmů indukce apoptózy nasycenými mastnými kyselinami (MK) a mechanizmů inhibice této indukce nenasycenými MK u pankreatických β-buněk.

Většina experimentů v rámci výzkumu apoptózy β-buněk je vzhledem k deficitu vhodných lidských β-buněčných linií prováděna na liniích krysích nebo myších, případně krysích nebo myších ostrůvcích. Lidské Langerhansovy ostrůvky se vzhledem k jejich dostupnosti a preferenčnímu použití pro transplantace používají minimálně. V našich experimentech jsme používali jednu z mála existujících lidských β-buněčných linií, a to NES2Y. Ta vznikla na Univerzitě v Leicesteru (UK) v roce 1997 po odebrání buněk z pacienta trpícího dětskou chronickou hyperinzulinemickou hypoglykémií (nasidioblastóza), (Macfarlane et al. 1997). Tyto buňky mají defektní expresi transkripčního faktoru IUF1 (insulin upstream factor 1) a proto nejsou schopny reagovat na glukózu. Nejsou tedy vhodné pro studie zabývající se procesy asociovanými se sekrecí inzulínu. Nicméně pro studium molekulárních mechanizmů apoptózy indukované MK není tato vlastnost limitující. Linie NES2Y byla již dříve použita pro studium indukce apoptózy v jiných laboratořích (Ou et al. 2005a, 2005b).

V publikacích, které jsou podkladem této práce, jsme používali 1 mM koncentraci nasycené kyseliny stearové (SA). Tato koncentrace SA se ukázala v našich předešlých experimentech jako nejvhodnější pro indukci apoptózy v pankreatických β-buňkách linie NES2Y nasycenými MK (Fürstová et al. 2008). 1 mM koncentrace SA zároveň koresponduje s chronicky zvýšenou hladinou SA, která je typická pro obézní pacienty. Fyziologická koncentrace SA se u dospělých pohybuje v rozmezí 0,110-1,170 mM (Lagerstedt et al. 2001, Abdelmagid et al. 2015). SA patří mezi nejčastěji zastoupené MK v krevní plazmě (Ingalls & Hoppel 1995, Lagerstedt et al. 2001, Stender et al. 2008). Vedle

SA jsme v pilotních pokusech testovali také vliv nasycené kyseliny palmitové (PA). Vzhledem k nižší účinnosti PA v indukci apoptózy pankreatických β-buněk jsme dále pracovali pouze s SA. V experimentech jsme dále používali nenasycenou kyselinu olejovou (OA) jakožto protějšek nasycené SA. Koncentrace 0,2 mM OA, která je fyziologická (Lagerstedt et al. 2001), se ukázala být v našich předešlých experimentech dostačující pro plnou inhibici indukce apoptózy nasycenou SA (1 mM), (Fürstová et al. 2008). Použité koncentrace MK se nacházejí v rozmezí koncentrací použitých v podobných experimentech jinými autory (např. Maedler et al. 2003, Eitel et al. 2002, Lupi et al. 2002). Vzhledem k tomu, že jsou MK ve vodě nerozpustné, byly v experimentech na buňky aplikovány vázané na sérový bovinní albumin. Fetální bovinní sérum, které je běžnou součástí používaných médií, obsahuje různé MK o nejasné koncentraci. My jsme proto v našich experimentech použili upravené bezsérové médium RPMI 1640 (Kovář & Franěk 1989, Koc et al. 2006), které umožňuje použít přesnou koncentraci dané MK. Nicméně media obsahující sérum jsou často v podobných experimentech používána (např. Maedler et al. 2001, Maestre et al. 2003, Welters et al. 2006, Diakogiannaki et al. 2007, Dhayal et al. 2008).

V experimentech, kde jsme sledovali vliv hypoxie, jsme jako silnou hypoxii používali 1% koncentraci kyslíku v kultivační atmosféře, jako střední hypoxii potom 4% koncentraci. 20% koncentrace kyslíku byla použita jako normoxie (ve vztahu k vnějším podmínkám, tj. koncentraci kyslíku ve vzduchu). Tyto, případně velmi podobné koncentrace kyslíku se v tomto smyslu běžně používají (Wang et al. 2012, Sato et al. 2014, Qiao et al. 2015).

6.2.1. Úloha kaspázy 2 v indukci apoptózy nasycenými mastnými kyselinami

Našemu pracovnímu kolektivu se podařilo jako prvnímu demonstrovat aktivaci kaspázy 2 nasycenými MK v β-buňkách (Němcová-Fürstová et al. 2011). Úloha kaspázy 2 v apoptóze pankreatických β-buněk byla prokázána již dříve v β-buněčné linii HIT-T15 v důsledku nedostatku GTP (Huo et al. 2002). Dále byla její aktivace demonstrována v lidských ostrůvcích v průběhu procesu odebírání a uchování pankreatu pro izolaci ostrůvků. Tato aktivace byla spojována s nižší viabilitou ostrůvků (Ramachandran et al. 2006). V jiné studii však nebyla prokázána úloha kaspázy 2 v regulaci viability pseudoostrůvků vytvořených β-buňkami linie MIN6 (Lock et al. 2011).

Kaspáza 2 se ukázala být klíčovou iniciační kaspázou aktivovanou v důsledku různých stimulů v řadě experimentálních modelů (Gu et al. 2008, Huang et al. 2007b,

Tiwari et al. 2011). Proto jsme předpokládali její případnou úlohu jako iniciační kaspázy i při indukci apopózy vyvolané nasycenými MK v pankreatických β-buňkách. Nezjistili jsme však žádný signifikantní efekt umlčení exprese kaspázy 2 prostřednictvím specifické siRNA (small interfering RNA) na viabilitu buněk NES2Y po aplikaci SA (viz publikace 1: obr. 6, str. 54). To naznačuje, že kaspáza 2 zde nemá významnou úlohu. Vzhledem k tomu, že jsme nezjistili ovlivnění aktivace ostatních kaspáz při umlčení exprese kaspázy 2 (viz publikace 1: obr. 7, str. 54), kaspáza 2 jejich aktivaci v buňkách linie NES2Y po aplikaci SA pravděpodobně nereguluje.

Předchozí práce naší laboratoře ukázala (Němcová-Fürstová et al. 2011), že hladina proteinu PIDD (regulační komponenta komplexu PIDDozómu) se po aplikaci MK nemění (Němcová-Fürstová et al. 2011). To naznačuje, že kaspáza 2 je zde velmi pravděpodobně aktivována mechanizmem nezávislým na formování komplexu PIDDozómu. Molekulární mechanizmy nezávislé na PIDDozómu již byly prokázány v některých jiných experimentálních modelech, kde byla apoptóza asociována s aktivací kaspázy 2 (např. Droin et al. 2001, Lavrik et al. 2006, Kim et al. 2009). Ukázalo se, že kaspáza 2 může být aktivována podobně jako kaspáza 8 i komplexem DISC (Droin et al. 2001, Lavrik et al. 2006, Olsson et al. 2009), ale nemusí se již dále podílet na přenosu proapoptotického signálu (Lavrik et al. 2006). K této situaci by mohlo docházet i v buňkách linie NES2Y, neboť zde dochází k aktivaci obou kaspáz a umlčení exprese kaspázy 2 nemělo vliv na proapoptickou signalizaci. Již dříve byla také prokázána aktivace kaspázy 2 kaspázou 7 (Inoue et al. 2009). K tomu by mohlo případně docházet i v buňkách linie NES2Y, neboť k aktivaci kaspázy 7 po aplikaci SA také dochází (viz publikace 1: obr. 4B, str. 53).

Ukázali jsme tak, že kaspáza 2 je v pankreatických β-buňkách NES2Y aktivována působením SA velmi pravděpodobně mechanizmem nezahrnujícím formování PIDDozómu. Kaspáza 2 v rámci aktivace kaspáz nepředstavuje apikální kaspázu a při apoptóze vyvolané nasycenými MK pravděpodobně nemá zásadní úlohu.

6.2.2. Úloha p38 MAPK a ERK signálních drah v indukci apoptózy nasycenými mastnými kyselinami a v inhibici této indukce nenasycenými mastnými kyselinami

6.2.2.1. Regulace aktivace p38 MAPK a ERK signálních drah mastnými kyselinami

Zjistili jsme, že SA v koncentraci indukující apoptózu (1 mM) v pankreatických β-buňkách NES2Y aktivuje p38 MAPK signální dráhu a inhibuje ERK signální dráhu (viz publikace

2: obr. 1C a 1D na str. 63). Naproti tomu nenasycená OA (0,2 mM), inhibuje uvedené účinky SA, přičemž sama o sobě ovlivňuje aktivaci testovaných drah pouze minimálně (viz publikace 4: obr. 2 na str. 96).

Aktivace p38 MAPK v důsledku samostatného působení PA a OA (vliv SA nebyl zjišťován), byla již na některých pankreatických β-buněčných liniích a ostrůvcích popsána (Cvjetićanin et al. 2009, Yuan et al. 2010, Natalicchio et al. 2013). Co se týče aktivace ERK signální dráhy, dostupné výsledky nejsou jednoznačné. V některých pracích byla po aplikaci PA prokázána aktivace (Fontés et al. 2008, Simon et al. 2008, Plaisance et al. 2009), v jiných inhibice (Guo et al. 2010, Watson et al. 2011) této dráhy. Aktivaci ERK kinázy po aplikaci SA ukázali pouze Simon et al. (2006). Zhang et al. (2007) pak prokázali aktivaci této kinázy po aplikaci OA. Nekonzistence dostupných dat může být do jisté míry způsobena odlišným experimentálním nastavení – doba působení MK a/nebo použité koncentrace se v některých studiích výrazně lišily. Dle nám dostupných poznatků nebyl inhibiční efekt nenasycených MK na aktivaci p38 MAPK a inhibici ERK signální dráhy, působením apoptózu indukujících koncentrací nasycených MK, dosud popsán.

Shrnutím našich a dalších dostupných výsledků můžeme konstatovat, že aplikace nasycených MK, v apoptózu indukující koncentraci, vede v pankreatických β-buňkách spíše k aktivaci p38 MAPK dráhy. Nenasycené MK tuto dráhu mohou aktivovat, pouze pokud jsou aplikovány ve vyšší koncentraci. Jinak jsou schopny inhibovat efekt nasycených MK. Vliv nasycených a nenasycených MK na aktivaci ERK dráhy je potom vzhledem k protichůdným výsledkům zatím nejasný.

6.2.2.2. Úloha p38 MAPK signální dráhy v indukci apoptózy nasycenými mastnými kyselinami

Zjistili jsme, že p38 MAPK signální dráha se v pankreatických β-buňkách linie NES2Y může podílet na apoptóze vyvolané 1 mM SA, ale pravděpodobně v tomto procesu nemá klíčovou úlohu (viz publikace 2: obr. 2 na str. 64, obr. 3 na str. 65, obr. 4 na str. 66 a obr. 5 na str. 67). K podobnému zjištění došli Yuan et al. (2010) na myší linii β-buněk NIT-1 s použitím nikoliv SA ale PA. Další autoři jejich výsledky v buňkách NIT-1 však nepotvrdili (Zhang et al. 2007a). Nicméně doba působení PA byla v jejich experimentu pouze 10 min. Naopak Abaraviciene et al. (2008), Cvjetićanin et al. (2009) a následně Natalicchio et al. (2013) naznačili nebo přímo ukázali (použitím specifického inhibitoru p38 MAPK) na krysích a/nebo lidských β-buněčných liniích a ostrůvcích, že p38 MAPK zprostředkovává

apoptózu indukovanou PA. Výsledky těchto autorů naznačují významnější úlohu p38 MAPK kinázy v procesu apoptózy vyvolané nasycenými MK.

Z výše uvedených publikovaných výsledků se zdá, že p38 MAPK aktivovaná nasycenými MK se v pankreatických β-buňkách pravděpodobně účastní proapoptotické signalizace vyvolané těmito kyselinami. Tato funkce p38 MAPK kinázy byla demonstrována i v hlodavčích a lidských ostrůvcích, které byly vystaveny oxidovaným lipidům (Ma et al. 2010) nebo cytokinům (Saldeen et al. 2001, Makeeva et al. 2006). Kromě toho, proapoptotická úloha této kinázy byla naznačena po aplikaci MK také v některých jiných typech buněk (Chai et al. 2007, Liu et al. 2009, Song et al. 2014).

6.2.2.3. Úloha ERK signální dráhy v indukci apoptózy nasycenými mastnými kyselinami

V naší práci jsme zjišťovali, zda p38 MAPK kináza reguluje aktivitu ERK signální dráhy. Ačkoliv některé z výsledků vztah mezi p38 MAPK a ERK signální dráhou neprokázaly (viz publikace 2: obr. 2B na str. 64 a obr. 4B na str. 66), celkově spíše naznačují, že p38 MAPK kináza inhibuje v pankreatických β-buňkách signální dráhu ERK (publikace 2: obr. 3B na str. 65 a obr. 5B na str. 67). V β-buňkách nebyl dosud vztah mezi uvedenými dráhami studován, ale v některých jiných typech buněk byl vliv p38 MAPK kinázy na dráhu ERK popsán (Berra et al. 1998, Lee et al. 2002). Tento vliv byl podobně jako v našem případě inhibičního charakteru.

Vzhledem k našemu zjištění, že p38 MAPK dráha pravděpodobně v procesu apoptózy buněk NES2Y indukované MK nemá klíčovou úlohu, tak pravděpodobně ani ERK signální dráha by v tomto procesu neměla hrát významnou roli. To však nevylučuje možnost, že v jiných typech buněk mohou mít obě dráhy významnější úlohu.

6.2.3. Úloha stresu ER v indukci apoptózy nasycenými mastnými kyselinami a v inhibici této indukce nenasycenými mastnými kyselinami

6.2.3.1. Regulace aktivace jednotlivých drah stresu ER mastnými kyselinami

Zjistili jsme, že SA v koncentraci vyvolávající apoptózu (1 mM) aktivuje všechny tři dráhy stresu ER (tj. IRE1α, PERK a ATF6 dráhy) již 3h po aplikaci (viz publikace 1: obr. 1 na str. 51, obr. 2 a 3 na str. 52). Tyto výsledky získané na lidské β-buněčné linii NES2Y korelují s výsledky získanými spíše na primárních β-buňkách krysího a lidského původu

(Cunha et al. 2008, Ladriere et al. 2010), než na hlodavčích β-buněčných liniích (Kharroubi et al. 2004, Cunha et al. 2008, Diakogiannaki et al. 2008).

Aktivace IRE1α dráhy v důsledku působení SA skrze detekci alternativního sestřihu XBP1 mRNA a aktivaci JNK (viz publikace 1: obr. 1 na str. 51 a obr. 2 na str. 52) byla prokázána i některými dalšími autory (Kharroubi et al. 2004, Cnop et al. 2007, Laybutt et al. 2007, Cunha et al. 2008, Gwiazda et al. 2009), ale ne všemi (Diakogiannaki et al. 2008, Lai et al. 2008, Ladriere et al. 2010). Tento zdánlivý rozpor by mohl být vysvětlen odlišnou mírou vyvolaného stresu ER v jednotlivých studiích, kdy se ukazuje, že IRE1α dráha je aktivována pouze v případě silného stresu ER (Lai et al. 2008). Analogický efekt SA na aktivaci PERK a fosforylaci eIF2α jako v buňkách NES2Y (viz publikace 1: obr. 1 na str. 51) byl zjištěn také v β-buněčných liniích zvířecího původu vystavených působení PA (Karaskov et al. 2006, Cnop et al. 2007, Laybutt et al. 2007, Cunha et al. 2008, Diakogiannaki et al. 2008, Lai et al. 2008, Gwiazda et al. 2009, Dhayal & Morgan 2011, Kim et al. 2018). Aplikace SA vyvolala v buňkách NES2Y aktivaci dráhy ATF6 (viz publikace 1: obr. 3, str. 52) obdobně jako v buňkách linie MIN6 působení PA (Laybutt et al. 2007). Je zajímavé, že v buňkách linie INS-1 aktivovala ATF6 dráhu OA (Kharroubi et al. 2004).

Nenasycená OA (0,2 mM) je schopna inhibovat účinky SA na IRE1α a PERK dráhy stresu ER, přičemž samotná OA ovlivňuje tyto dráhy minimálně. To bylo zjištěno jak námi u lidské β-buněčné linii NES2Y (viz publikace 4: obr. 3 na str. 97, obr. 4 na str. 98 a obr. 5 na str. 99) tak u krysích linií BRIN-BD11 a INS-1E (Diakogiannaki et al. 2008, Sommerweiss et al. 2013). Co se týče vlivu působení OA na efekt SA na aktivaci ATF6 dráhy, nejsou dostupná žádná data.

Můžeme tedy shrnout, že aplikace nasycených MK, v koncentraci indukující apoptózu, vede k silné aktivaci všech tří signálních drah stresu ER. Nenasycené MK jsou schopny inhibovat aktivující účinek nasycených MK na IRE1α a PERK dráhy stresu ER a samy ovlivňují jejich aktivaci pouze minimálně. Vliv nenasycených MK na efekt nasycených MK na aktivaci ATF6 dráhy nebyl zatím nikým studován. Je dostupná pouze jedna práce, kde aplikace (samotné) nenasycené OA vedla k aktivaci dráhy ATF6.

6.2.3.2. JNK a indukce stresu ER nasycenými mastnými kyselinami

Inhibice aktivity JNK snížila upregulaci proteinu BiP způsobenou SA (viz publikace 1: obr. 4A, str. 53). JNK kináza se tedy na regulaci stresu ER určitým způsobem podílí, pravděpodobně prostřednictvím regulace aktivace „downstream“ od proteinu c-Jun. c-Jun

se může účastnit procesu formování komplexu AP-1 (activator protein-1), který vedle exprese ATF6 a XBP1 reguluje také expresi BiP proteinu (He et al. 2000, Yamamoto et al. 2004). Toto vysvětlení není v souladu s minimálním efektem JNK inhibice na expresi CHOP (viz publikace 1: obr. 4A, str. 53), který je také regulován komplexem AP-1 (Pirot et al. 2007). Kromě toho bylo již dříve v buňkách linie INS-1 prokázáno, že na formování komplexu AP-1 se po aplikaci PA nepodílí c-Jun ale Jun-B (Pirot et al. 2007). Jun-B je další člen Jun rodiny transkripčních faktorů, jejichž aktivace je nezávislá na JNK (Kallunki et al. 1996).

JNK kináza je považována za případný spojující článek mezi stresem ER a indukcí apoptózy. Nicméně, po specifické inhibici této kinázy inhibitorem SP600125, jsme nezjistili inhibici či zpoždění aktivace kaspáz ani zvýšení exprese CHOP (viz publikace 1: obr. 4A a 4B, str. 53) jak ukázali Cunha et al. (2008). Naše výsledky naznačují, že JNK není v buňkách NES2Y mediátorem apoptózy vyvolané SA, nelze však vyloučit možnou „prosurvival“ úlohu JNK kinázy. Ta byla některými autory prokázána (Park et al. 2007, Leung et al. 2008).

JNK kináza se tedy v pankreatických β-buňkách pravděpodobně určitým způsobem podílí na regulaci stresu ER. Její úloha coby mediátoru mezi stresem ER a indukcí apoptózy vyvolané nasycenými MK není však jasná.

6.2.3.3. Kaspáza 2 a indukce stresu ER

Zjistili jsme, že umlčení exprese kaspázy 2 snížilo zvýšenou hladinu proteinu BiP po aplikaci SA (viz publikace 1: obr. 8, str. 55). Logette et al. (2005) ukázali, že exprese kaspázy 2 může být transkripčně regulována proteiny SREBP (sterol-regulatory element binding protein) 2 a SREBP1c, což jsou hlavní aktivátory genů kódujících proteiny regulující syntézu cholesterolu a MK. Tito autoři dále zjistili, že umlčení exprese kaspázy 2 vede v buňce k poklesu hladin cholesterolu a triacylglycerolu (Logette et al. 2005). Kromě toho, Zhang et al. (2007b) ukázali, že myši s deficitem kaspázy 2 (knockout) mají méně tělesného tuku než normální myši. Zdá se tedy, že kaspáza 2 se může určitým způsobem podílet na signalizaci regulující metabolismus lipidů, což může následně ovlivnit míru stresu ER v buňkách.

6.2.4. „Upstream“ mechanizmy indukce apoptózy nasycenými mastnými kyselinami a místo inhibiční intervence nenasycených mastných kyselin

Výsledky našich (obr. 3, str. 117) i některých dalších studií (Vacaresse et al. 1999, Holzer et al. 2011) poukazují na to, že počátek dráhy indukce apoptózy nasycenými MK se nachází „upstream“ od námi studovaných signálních drah p38 MAPK, ERK a stresu ER. Je možné, že signalizace vyvolaná MK může začínat již na plazmatické membráně buňky, kde MK ovlivňují její fluiditu. Nasycené MK s rigidním a rovným acylovým řetězcem po inkorporaci do membránové dvojvrstvy snižují membránovou fluiditu (Karnovski et al. 1982). To může změnit schopnost membránových receptorů (např. receptorových tyrosin kináz) dimerizovat a tedy přenášet signály dále. Signály tohoto typu mohou mít charakter proliferační a/nebo„prosurvival“.

Nenasycené MK s „ohnutým“ acylovým řetězcem pak redukují membránovou fluiditu výrazně méně než nasycené MK či jsou schopny ji dokonce zvyšovat. Co se týče možného mechanizmu inhibiční intervence nenasycených MK v signalizaci vyvolané nasycenými MK, tak jeden z možných mechanizmů může být zvýšení membránové fluidity a tím kompenzování efektu nasycených MK na rigiditu membrány. Dalo by se také spekulovat o možné úloze lepší schopnosti nenasycených MK se inkorporovat do plazmatické membrány v průběhu „kompetice“ s nasycenými MK. Nicméně, abychom potvrdili tyto spekulace, jsou potřeba další specifické studie.

6.2.5. Vliv hypoxie na indukci apoptózy nasycenými mastnými kyselinami a na inhibici této indukce nenasycenými mastnými kyselinami

6.2.5.1. Vliv hypoxie na indukci apoptózy a na inhibici této indukce mastnými kyselinami

Ukázali jsme, že hypoxie (střední i silná) signifikantně zvyšuje proapoptotický efekt 1 mM SA, přičemž efekt silné hypoxie byl výrazně větší než efekt střední hypoxie (viz publikace 5: obr. 1, str. 108). Tyto výsledky naznačují, že když se působení hypoxie kombinuje s působením jiného proapoptotického faktoru, hypoxie pak může představovat rozhodující element vedoucí k indukci apoptózy pankreatických β-buněk. Potenciace cytotoxického efektu různých látek v důsledku hypoxie byla zjištěna v jiných typech buněk (Walford et al. 2004, Bullova et al. 2016).

Dále jsme ukázali, že hypoxie signifikantně snížila protektivní efekt 0,2 mM OA proti proapoptotickému efektu SA a to takovým způsobem, že OA již nebyla schopna

blokovat indukci apoptózy β-buněk vyvolanou působením SA. Efekt silné hypoxie byl opět výraznější než efekt střední hypoxie (viz publikace 5: obr. 1, str. 108). Zdá se tedy, že hypoxie může v pankreatických β-buňkách také inhibovat antiapoptotický efekt některých látek. Zatím nebyly publikovány žádné práce zabývající se tímto tématem.

Zjistili jsme, že střední hypoxie (4 % O_2) nemá žádný efekt na růst a viabilitu lidských pankreatických buněk NES2Y, zatímco silná hypoxie (1% O_2) má určitý relativně mírný účinek snižující růst buněk (viz publikace 5: obr. 1, str. 108). Je zajímavé, že na β-buňky hlodavčích linií (INS-1, MIN6) má silná, ale i střední hypoxie výrazně silnější proapoptotický efekt (Lai et al. 2009, Zheng et al. 2012, Sato et al. 2014, Qiao et al. 2015). To by mohlo naznačovat, že lidské pankreatické β-buňky jsou vůči hypoxickému stresu odolnější než β-buňky hlodavčí. Zda je to dáno narušením mechanizmů zprostředkovávajících poškozující účinek hypoxie v linii NES2Y nebo jde o fyziologickou situaci u lidských β-buněk, zůstává otázkou.

Hypoxie tedy může představovat klíčový faktor rozhodující o přežívání/smrti pankreatických β-buněk v přítomnosti MK a tedy v důsledku i o vzniku diabetu 2. typu. Žádná jiná studie zabývající se vlivem spolupůsobení hypoxie a MK nebyla dosud publikována.

6.2.5.2. Mechanizmy hypoxie a místo intervence hypoxie do mechanizmů indukce apoptózy a inhibice této indukce mastnými kyselinami

Co se týče molekul potenciálně zprostředkovávajících poškozující účinek hypoxie v buňkách NES2Y, naše výsledky naznačují (viz publikace 5: obr. 2, str. 109), že došlo k aktivaci jak iniciačních (kaspáza 8 a 9), tak exekučních kaspáz (kaspáza 6 a 7). Dosud bylo v pankreatických β-buňkách zjištěno pouze zapojení kaspázy 8, 3 a 7 jakožto mediátorů poškozujícího účinku hypoxie (Lai et al. 2009, Qiao et al. 2015, Sato et al. 2014, Zheng et al. 2012, Wang et al. 2012, Bensellam et al. 2016, Ryu et al. 2009, Tian et al. 2013). My jsme však nově prokázali možné zapojení kaspázy 9 a 6. Je zajímavé, že aktivace kaspáz vyvolaná působením nasycených MK byla snížena za středně hypoxických podmínek (viz publikace 5: obr. 2, str. 108), přičemž ale nedošlo k zvýšení počtu přežívajících buněk. Naopak jsme zjistili snížení počtu přežívajících buněk (viz publikace 5: obr. 2, str. 109). Vysvětlení těchto protichůdných výsledků je značně problematické.

Zjistili jsme, že hypoxie potenciuje efekt MK na expresi (její zvýšení) hlavních markerů stresu ER, proteinů BiP a CHOP (viz publikace 5: obr. 3, str. 110). Tyto výsledky stejně jako výsledky dalších autorů (Oslowski & Urano 2011, Zheng et al. 2012) naznačují,

že vedle aktivace kaspáz zprostředkovává v pankreatických β-buňkách proapoptotický účinek hypoxie také signalizace stresu ER. Bylo prokázáno, že signalizace stresu ER se nalézá „upstream“ od aktivace kaspáz (Tabas & Ron 2011). Proto se musí intervence hypoxie do mechanizmů indukce apoptózy nasycenými MK a do mechanizmů inhibice této indukce nenasycenými MK nalézat v rámci nebo „upstream“ od drah stresu ER.

ER stres vyvolaný působením MK je v β-buňkách potenciován hypoxií pravděpodobně proto, protože proces „sbalování“ molekul proinzulínů v ER zahrnuje vytvoření tří disulfidových vazeb reakcí vyžadující molekulární kyslík (Appenzeller-Herzog & Ellgaard 2008). Když je však molekulárního kyslíku nedostatek, dochází k akumulaci nesbalených molekul proinzulínu stejně jako dalších proteinů. To vede ke snížení funkce β-buněk a dohromady s působením MK přispívá k narušení homeostáze ER. V srdečních myocytech bylo zjištěno, že silná hypoxie vyvolává redistribuci dvou transportérů MK (FAT/CD36 a FABPpm) z cytoplazmy na plazmatickou membránu buňky. To vede k zvýšení příjmu MK do buňky (Chabowski et al. 2006). Oba transportéry se nacházejí i v pankreatických β-buňkách (Hyder et al. 2010, Dalgaard et al. 2011). K této redistribuci v důsledku hypoxie by mohlo docházet obdobně i v β-buňkách a tedy by tento jev mohl představovat mechanizmus, kterým hypoxie v pankreatických β-buňkách zvyšuje proapoptotický efekt nasycených MK. Vzhledem k tomu, že nenasycená OA může mít v β-buňkách při vyšších koncentracích také proapoptotický efekt (Cnop et al. 2002, Cunha et al. 2008, Tuo et al. 2011) uvedený zvýšený příjem MK v důsledku redistribuce transportérů MK by mohl znamenat, že OA již dále nepůsobí cytoprotektivně, ale naopak může β-buňkám škodit. A/Nebo, OA již není schopna inhibovat proapototické působení SA, jejíž koncentrace se zvýšila v důsledku hypoxií vyvolané redistribuce transportérů MK.

Ukazuje se tak, že hypoxie vede v pankreatických β-buňkách k zapojení drah stresu ER. Místo intervence proapoptotického efektu hypoxie do mechanizmů indukce apoptózy a inhibice této indukce MK se musí nalézat „upstream“ nebo v rámci drah stresu ER.

7. PUBLIKACE

7.1. PUBLIKACE 1

Němcová-Fürstová V, **Šrámek J**, Balušíková K, James RF, Kovář J:
Caspase-2 and JNK activated by saturated fatty acids are not involved in apoptosis induction but modulate ER stress in human pancreatic β-cells.
Cellular Physiology and Biochemistry, 31: 277–289, 2013.

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013
Accepted: February 07, 2013

Karger Open access

277

1421-9778/13/0313-0277$38.00/0

Original Paper

Caspase-2 and JNK Activated by Saturated Fatty Acids are Not Involved in Apoptosis Induction but Modulate ER Stress in Human Pancreatic β-cells

Vlasta Němcová-Fürstová[a] Kamila Balušíková[a] Jan Šrámek[a] Roger F. James[b] Jan Kovář[a]

[a]Division of Cell and Molecular Biology & Center for Research of Diabetes, Metabolism and Nutrition, Third Faculty of Medicine, Charles University, Prague, [b]Department of Infection, Immunity and Inflammation, University of Leicester, Leicester

Key Words
Caspase-2 • JNK • Saturated fatty acids • Apoptosis • Endoplasmic reticulum stress • β-cells

Abstract
Background: Fatty acid-induced apoptosis and ER stress of pancreatic β-cells contribute to the development of type 2 diabetes, however, the molecular mechanisms involved are unclear. ***Aims:*** In this study we have tested the role of caspase-2 and suggested ER stress mediator JNK in saturated fatty acid-induced apoptosis of the human pancreatic β-cells NES2Y. ***Results:*** We found that stearic acid at apoptosis-inducing concentration activated ER stress signaling pathways, i.e. IRE1α, PERK and ATF6 pathways, in NES2Y cells. During stearic acid-induced apoptosis, JNK inhibition did not decrease the rate of apoptosis nor the activation of caspase-8, -9, -7 and -2 and PARP cleavage. In addition, inhibition of JNK activity did not affect CHOP expression although it did decrease the induction of BiP expression after stearic acid treatment. Caspase-2 silencing had no effect on PARP as well as caspase-8, -9 and -7 cleavage and the induction of CHOP expression, however, it also decreased the induction of BiP expression. Surprisingly, caspase-2 silencing was accompanied by increased phosphorylation of c-Jun. ***Conclusions:*** We have demonstrated that caspase-2 as well as JNK are not key players in apoptosis induction by saturated fatty acids in human pancreatic β-cells NES2Y. However, they appear to be involved in the modulation of saturated fatty acid-induced ER stress signaling, probably by a mechanism independent of c-Jun phosphorylation.

Introduction

There is convincing experimental evidence showing that saturated fatty acid exposure (in contrast to unsaturated fatty acids) is detrimental to pancreatic β-cell survival and leads to β-cell apoptosis by still unclear molecular mechanisms [1-3].

Jan Kovář

Division of Cell and Molecular Biology, Third Faculty of Medicine, Charles University, Ruská 87, 100 00 Prague 10 (Czech Republic)
Tel. +420/267102658, Fax +420/267102650, E-Mail jan.kovar@lf3.cuni.cz

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013

Němcová-Fürstová/Balušíková/Šrámek/James/Kovář: Caspase-2 and JNK in Fatty Acid-Induced Apoptosis

As secretory cells, β-cells are especially prone to perturbations in endoplasmic reticulum (ER) function [4] and subsequent triggering of ER stress. Saturated fatty acids were shown to activate ER stress signaling in pancreatic β-cells *in vitro* [3, 5-8] and increased levels of ER stress markers have been demonstrated in β-cells of type 2 diabetes patients [9-11]. Therefore, ER stress is suggested as a likely mechanism that mediates the pro-apoptotic effect of saturated fatty acids in pancreatic β-cells [12, 13].

It has been proposed that ER stress signaling is mediated by three sensor proteins localized in the ER membrane, i.e. IRE1α (inositol-requiring protein 1α), PERK (protein kinase RNA (PKR)-like ER kinase) and ATF6 (activating transcription factor 6). Activation of IRE1α leads to JNK (c-Jun N-terminal kinase) activation by phosphorylation and to unconventional splicing of mRNA for XBP1 (X-box binding protein 1) which results in translation of active transcription factor (XBP1s). Activation of PERK results in the inhibition of protein translation via phosphorylation of eIF2α (eukaryotic initiation factor 2α). When the ATF6 pathway is activated, ATF6 translocates to nucleus where it functions as transcription factor. All this signaling is aimed primarily at the restoration of ER homeostasis, e.g. by decreasing protein translation and increasing the expression of chaperones, such as the prominent ER chaperone BiP (immunoglobulin heavy chain-binding protein) [14]. However, if this response fails, apoptosis is induced by the mechanisms that are not still completely understood. The proposed mediators are JNK and transcription factor CHOP (CCAAT-enhancer-binding protein (C/EBP) homologous protein) [15].

Within the framework of ER stress signaling, JNK is supposed to exert its effect via modulation of the activity of several Bcl-2 family proteins by phosphorylation and via phosphorylation of c-Jun that participates in formation of AP-1 (activator protein-1) complex involved in transcriptional regulation of many predominantly pro-apoptotic genes, including CHOP [16]. Fatty acid treatment activates JNK in β-cells [6, 17-19] and it is known that type 2 diabetes is associated with the activation of the JNK pathway [20], however, the contribution of JNK in fatty acid-induced apoptosis and ER stress in β-cells is not clear.

In our previous research we have newly demonstrated that caspase-2 is activated together with ER stress induction when apoptosis is induced by saturated fatty acids in human pancreatic β-cell line NES2Y [8, 21]. There are also several other lines of evidence pointing at caspase-2 as a possible transducer of pro-apoptotic ER stress signaling in various cell types [22-25] although the mode of its activation under ER stress conditions is rather speculative.

In the present study, we have tested the role of caspase-2 and suggested ER stress mediator JNK in saturated fatty acid-induced apoptosis and ER stress signaling in human pancreatic β-cells NES2Y. Here we demonstrate that caspase-2, as well as JNK, are not key players in apoptosis induction by saturated fatty acids in pancreatic β-cells. However, JNK appears to be involved in the modulation of saturated fatty acid-induced ER stress signaling.

Materials and Methods

Materials

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated. For western blot analysis and fluorescence microscopy, the following primary and secondary antibodies were used: anti-BiP (#3177), anti-CHOP (#2895), anti-cleaved caspase-7 (#9491), anti-cleaved caspase-8 (#9496), anti-cleaved caspase-9 (#9505), anti-phospho eIF2α (#9721), anti-phospho-c-Jun (#9261), anti-c-Jun (#9165), anti-phospho-SAPK/JNK (#4668), anti-SAPK/JNK (#9258) and anti-PARP (#9542) from Cell Signaling Technology (Danvers, MA, USA), anti-actin (clone AC-40) from Sigma-Aldrich (St. Louis, MO, USA), anti-ATF6 (ab 11909) and anti-caspase-2 (ab 32021) from Abcam (Cambridge, UK), HRP-linked goat anti-mouse and goat anti-rabbit antibody from Santa Cruz (Santa Cruz, CA, USA) and AlexaFluor 488-conjugated goat anti-mouse antibody from Invitrogen (Invitrogen-Molecular Probes, Eugene, OR, USA).

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013

Cells and culture conditions

The human pancreatic β-cell line NES2Y [21, 26] was used. NES2Y are proliferating insulin-secreting cells with a defect in glucose responsiveness. Cells were routinely maintained in an RPMI 1640 based culture medium [27]. In experiments, a defined serum-free medium [28] supplemented with stearic acid bound to 2% fatty-acid free bovine serum albumin (BSA) was used [21]. Stock solution containing stearic acid bound to the 10% BSA in the serum-free medium was prepared as described previously [21] and diluted to the required concentration of fatty acid and BSA prior to experiments. Fatty acid/BSA molar ratios used in experiments were lower than the ratios known to exceed the binding capacity of BSA [29]. In some experiments, thapsigargin at a concentration of 1 μM in serum-containing culture medium was used for ER stress induction.

Flow cytometric assessment of cell viability

Cells (approximately $5x10^5$ cells per sample) were seeded and stearic acid or control media were applied after 24-h preincubation as described above (see "Western blot analysis"). After 18 and 24-h incubation, the percentage of viable, apoptotic and necrotic cells was determined employing flow cytometry and Annexin V-FITC Apoptosis Detection Kit (Abcam, Cambridge, UK) according to manufacturer´s instructions. Apoptotic cells were defined as annexin V-FITC positive cells. Necrotic cells were defined as propidium iodide-positive, annexin V-FITC negative cells.

Western blot analysis

Cells (approximately $1-3x10^6$ cells per sample) were seeded and after a 24-h preincubation period allowing cells to attach, the culture medium was replaced by the serum-free medium containing stearic acid or thapsigargin at required concentration or by control medium. After required period of incubation, both adherent and floating cells were harvested and western blot analysis was performed as described previously [8]. All primary antibodies were used in 1:1 000 dilution with exception of anti-CHOP antibody that was diluted 1:500. The chemiluminiscent signal was detected using Carestream Gel Logic 4000 PRO Imaging System equipped with Carestream Molecular Imaging Software (Carestream Health, New Haven, CT, USA) for image acquisition and analysis.

Assessment of XBP1 mRNA splicing

Cells (approximately $1x10^6$ cells per sample) were seeded and stearic acid and thapsigargin were applied after 24-h preincubation as described above (see "Western blot analysis"). After 3, 6, 12 and 24-h incubation, the cells were harvested and the splicing of XBP1 mRNA was assessed by RT-PCR as described previously [8, 30] with GAPDH as a housekeeping gene [31].

Confocal microscopy analysis of ATF6 translocation

Cells were seeded onto coverslips (approximately 2×10^5 cells per coverslip) and stearic acid or thapsigargin were applied after 24-h preincubation as described above (see "Western blot analysis"). After desired period of induction, coverslips were fixed with 4% paraformaldehyde for 20 min at 37°C. After washing with PBS, cells were permeabilized with 0.1% Tween in PBS for 10 min and blocked with FX Enhancer (Invitrogen-Molecular Probes, Eugene, OR, USA). ATF6 was stained employing primary antibody against ATF6 (Abcam, Cambridge, UK) (1:50 in PBS at 4°C overnight) and AlexaFluor 488-conjugated goat anti-mouse secondary antibody (Invitrogen-Molecular Probes, Eugene, OR, USA) (1:100 in PBS for 2 hour in the dark at room temperature). Stained cells on coverslips were transfered onto a droplet of Vectashield® Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) or ProLong Gold with DAPI (Invitrogen-Molecular Probes, Paisley, UK) and sealed. Samples were analyzed employing a confocal microscope Leica TCS SP5 (Bannockburn, IL, USA) with 63x oil objective at relevant excitation and emission wavelengths.

Inhibition of JNK kinase activity

Cells (approximately $1x10^6$ cells per sample) were seeded and after a 23-h preincubation period allowing cells to attach, the culture medium was replaced by the serum-free medium (for stearic acid treatment) or serum-containing medium (for thapsigargin treatment) with JNK inhibitor SP600125 (Enzo Life Sciences, Farmingdale, NY, USA) at desired concentration, or by control medium with the vehiculum

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013

only (DMSO). After 1 h of pretreatment with JNK inhibitor, stearic acid and BSA or thapsigargin were added to achieve the required concentrations. After 18 h and 24 h of incubation the cells were harvested and lysates were prepared for Western blot analysis.

Assessment of the effect of JNK inhibition on growth and viability

Cells were seeded at 2 x 10^4 cells/100 µl of culture media (see above) into wells of 96-well plate. The JNK inhibitor SP600125 and stearic acid were applied in the same way as described in „Inhibition of JNK kinase activity". After 48 h-incubation, the number of living cells was determined by hemocytometer counting after staining with trypan blue.

Silencing of caspase-2 expression by siRNA

For the inhibition of caspase-2 expression, caspase-2 specific siRNA (Applied Biosystems, Foster City, CA, USA) and INTERFERin (PolyPlus-Transfection, Illkirch, France) as transfection reagent, were used according to manufacturer instructions. Nonspecific siRNA (Applied Biosystems, Foster City, CA, USA) was used as negative control. The efficiency of inhibition of caspase-2 expression at the protein level was tested after 72 or 96 h of siRNA treatment in each experiment by Western blot analysis of caspase-2 expression.

For caspase-2 silencing, 1.5 x 10^5 cells were seeded into Petri dishes (Ø 6 cm). After 24 h allowing cells to attach, the media was changed for media containing caspase-2 specific siRNA or nonspecific siRNA at final concentration of 5 nM and INTERFERin transfection reagent at 1:300 dilution. After 72 h of incubation with siRNA, cells were harvested into their cultivation media and seeded in the same media into 6-well plates in concentration of 1 x 10^6 cells/2.5 ml per well. After 24 h allowing cells to attach, the media was replaced by relevant media for the treatment with stearic acid or thapsigargin, containing in addition fresh siRNA and transfection reagent at the same concentration as used for the initial inhibition of caspase-2 expression. Stearic acid- or thapsigargin-induced and control cells were harvested after 6, 18 and 24 h of treatment as described in „Western blot analysis". The level of caspase-2 expression during each experiment was tested after 72 h of induction with siRNA, i.e. at the time of seeding cells for stearic acid or thapsigargin application, and at the end of experiment, i.e. 24 h after the application.

Assessment of the effect of caspase-2 silencing on growth and viability

The experiment was set up exactly in the same way as described in „Inhibition of caspase-2 expression by siRNA", but after 72 h-incubation with siRNA, nontransfected and both caspase-2 siRNA and nonspecific siRNA treated cells were seeded at 2 x 10^4 cells/100 µl of relevant media (see above) into wells of 96-well plate and after 48 h-incubation with stearic acid, the number of living cells was determined by hemocytometer counting after staining with trypan blue.

Statistical analysis

Statistical significance of differences was determined by Student´s *t*-test. $P < 0.05$ was considered statistically significant.

Results

Assessment of the mode of stearic acid-induced cell death

Our previous studies showed that stearic acid at a concentration of 1 mM induces cell death in most of NES2Y cells within 48 h after stearic acid application. However, apoptotic cells as well as caspase-2 activation appeared within 24 h after the application [8, 21, our unpublished data]. Therefore, all effects were tested until 24 h after fatty acid application. Under the experimental conditions used, application of stearic acid (1 mM) led to apoptosis in 16.2 ± 0.8 % of cells and necrosis in 3.2 ± 0.2 % of cells within 18 h after the application. In corresponding controls it was 2.9 ± 0.1 % and 0.8 ± 0.1 %, respectively. Within 24 h after the application, it led to apoptosis in 28.3 ± 7.6 % of cells and necrosis in 6.3 ± 0.2 % cells. In corresponding controls it was 3.8 ± 0.1 % and 1.6 ± 0.1 %, respectively, as assessed by flow cytometry after annexin V-propidium iodide staining (data not shown).

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013

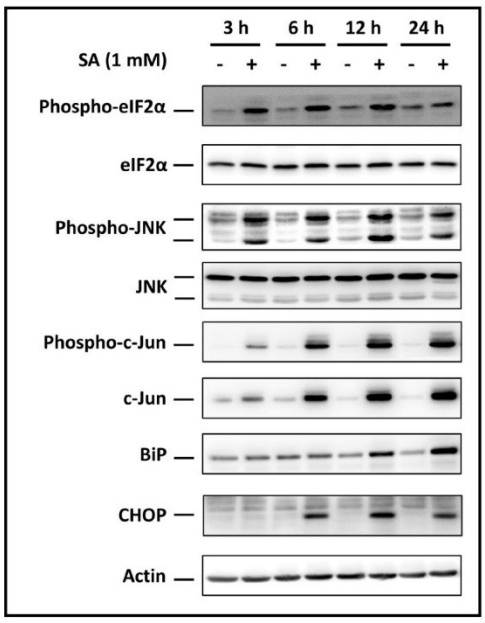


Fig. 1. Effect of 1 mM stearic acid (SA) on the expression of phospho-eIF2α, eIF2α, phospho-JNK, JNK, phospho-c-Jun, c-Jun, BiP and CHOP in NES2Y cells. Cells incubated without stearic acid represented control cells. After 3, 6, 12 and 24 h of incubation, the expression of proteins was determined by western blot analysis employing relevant antibodies (see „Materials and Methods"). Monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment of at least three independent experiments.

Activation of ER stress signaling by stearic acid

We tested the level of expression of molecules known to be involved in ER stress signaling (phospho-eIF2a, phospho-JNK, phospho-c-Jun, Bip and CHOP) at 3, 6, 12 and 24 h after stearic acid (1 mM) treatment of NES2Y cells. Interestingly, we found that JNK (IRE1α pathway of ER stress signaling) was phosphorylated and thus activated as early as 3 h after stearic acid application. This activation was accompanied by increased expression and phosphorylation of transcription factor c-Jun, i.e. the target of JNK activity. Phosphorylation of both these molecules lasted until 24 h after the application (Fig. 1). Stearic acid also induced early splicing (3 h after the application) of XBP1 (Fig. 2).

To assess the involvement of PERK pathway of ER signaling, we tested the phosphorylation status of the translation factor eIF2α. Stearic acid treatment resulted in early phosphorylation (3 h after the application) of eIF2α that lasted at least for the first 12 hours of treatment but then decreased to the level comparable with control cells in contrast to the molecules involved in IRE1α signaling, i.e. JNK and c-Jun.

We employed confocal microscopy in order to assess ATF6 translocation to the nucleus after its activation by cleavage, as it has been demonstrated after palmitate application in pancreatic β-cells previously [11]. As a positive control, cells were treated with thapsigargin, a synthetic inductor of ER stress. We were able to detect ATF6 translocation due to stearic acid application as early as 3 h after the application (Fig. 3). This activation lasted at least 12 h. After 24 h of treatment, the assessment of the extent of ATF6 translocation after stearic acid application was very unreliable due to undergoing apoptosis.

As to the downstream effector molecules we found CHOP expression to be induced 6 h after stearic acid application that lasted for 24 h (Fig. 1). In contrast, we did not observe any change in BiP level until 12 h of stearic acid treatment. Significant increase in BiP level was detected 12 h and 24 h after stearic acid application (Fig. 1).

Effect of JNK inhibition on ER stress

We assessed the role of JNK in stearic acid-induced ER stress and apoptosis in NES2Y β-cells employing SP600125, a specific inhibitor of JNK. The concentration of inhibitor, which was necessary for efficient JNK inhibition, was determined by testing the effect of several inhibitor concentrations on the level of phosphorylated c-Jun (data not shown).

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013

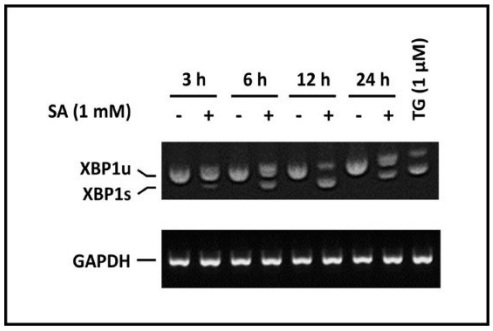


Fig. 2. Effect of 1 mM stearic acid (SA) on XBP1 mRNA splicing in NES2Y cells. Cells incubated without stearic acid represented control cells. After 3, 6, 12 and 24 h of incubation, the XBP1 splicing was assessed by RT-PCR using relevant primers (see „Materials and Methods"). As a positive control of XBP1 splicing, NES2Y cells were treated with 1 µM thapsigargin (TG). GAPDH was used as a control gene for RT-PCR. The data shown were obtained in one representative experiment of three independent experiments.

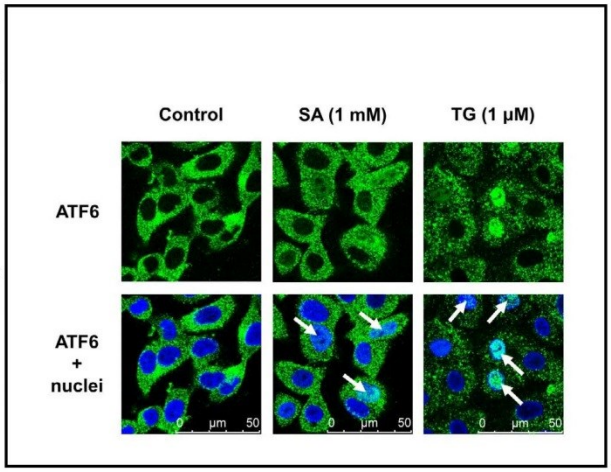


Fig. 3. Effect of 1 mM stearic acid (SA) on ATF6 translocation into nucleus in NES2Y cells. Cells incubated without stearic acid represented control cells. After 3 h of incubation, ATF6 (green) and nuclei (blue) within the cells were stained and representative images were acquired with Leica confocal scanning microscope (see „Materials and Methods"). As a positive control of ATF6 translocation, NES2Y cells were treated with 1 µM thapsigargin (TG). ATF6 staining and the merge of ATF6 and nuclear signal is shown. Data obtained in one representative experiment of three independent experiments are shown.

Using 50 and 100 µM SP600125 together with stearic acid, we achieved 61±5% and 82±7% decrease of phosphorylated c-Jun level, respectively, after 24 h of treatment compared to cells treated with stearic acid alone. Inhibition of JNK activity in stearic acid-treated cells resulted in significant decrease of BiP level after 18 h and 24 h of treatment. However, surprisingly CHOP expression induced by stearic acid was not affected by JNK inhibition (Fig. 4A, only data for 18 h are shown). To determine the role of JNK inhibition in the regulation of BiP expression, we assessed the effect of JNK inhibition on BiP upregulation induced by another ER stressor, i.e. thapsigargin. There was only slight effect of JNK inhibition on BiP expression after 18 h of treatment with 1 µM thapsigargin, however, the inhibitory effect on BiP expression was clearly detectable after shorter period (6 h) of treatment. Similarly to the effect of JNK inhibition after stearic acid application, CHOP expression induced by thapsigargin was not affected by JNK inhibition, (Fig. 5).

Effect of JNK inhibition on caspase activation and apoptosis induction

We showed previously that caspase-7, caspase-9, caspase-8 and caspase-2 are activated during stearic acid-induced apoptosis in NES2Y β-cells, in contrast to caspase-3. Therefore, we tested the effect of inhibition of JNK activity on activation (cleavage) of these caspases and also on the cleavage of PARP (common marker of apoptosis) after 18 and 24 h of treatment. However, the level of cleaved caspase-7, caspase-9, caspase-8 and PARP and the

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013

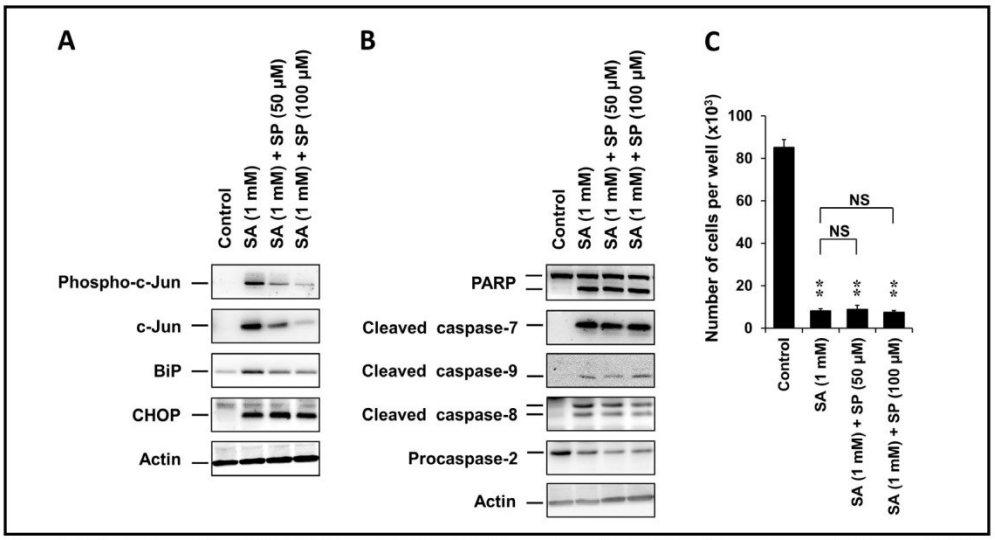
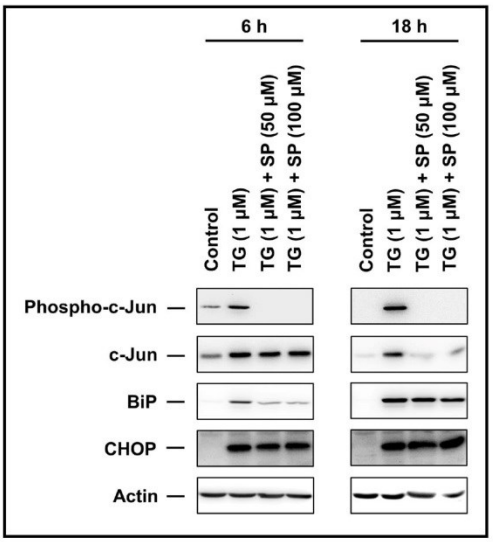


Fig. 4. Effect of specific JNK inhibitor SP600125 (SP) on the effect of 1 mM stearic acid (SA) on (A) the expression of phospho-c-Jun, c-Jun, BiP and CHOP, on (B) the cleavage of PARP, caspase-7, caspase-9, caspase-8 and caspase-2 in NES2Y cells and on (C) the cell growth and viability of NES2Y cells. Cells incubated without stearic acid represented control cells. After 18 h (A) or 24 h (B) of incubation, the expression or cleavage of proteins was determined by western blot analysis employing relevant antibodies (see „Materials and Methods"). Monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment of three independent experiments. (C) Cells were seeded at $2x10^4$ cells/100 µl of culture medium per well of 96-well plate and stearic acid and SP600125 were applied as described in "Materials and Methods". The number of living cells was determined after 48 h of incubation. Each column represents the mean of 4 separate cultures ± SEM. ** $P<0.01$ when comparing the effect of stearic acid with or without SP600125 and control cells, nonsignificant (NS) when comparing the effect of stearic acid applied together with SP600125 and the treatment with stearic acid alone.

Fig. 5. Effect of specific JNK inhibitor SP600125 (SP) on the effect of 1 µM thapsigargin (TG) on the expression of phospho-c-Jun, c-Jun, BiP and CHOP in NES2Y cells. Cells incubated without thapsigargin represented control cells. After 6 h and 18 h of incubation, the expression of proteins was determined by western blot analysis employing relevant antibodies (see „Materials and Methods"). Monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment of three independent experiments.



level of procaspase-2 were not influenced by JNK inhibition during stearic acid treatment (Fig. 4B, only data for 18 h are shown) as well as the cell growth and viability within 48 h of treatment (Fig. 4C).

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013

Němcová-Fürstová/Balušíková/Šrámek/James/Kovář: Caspase-2 and JNK in Fatty Acid-Induced Apoptosis

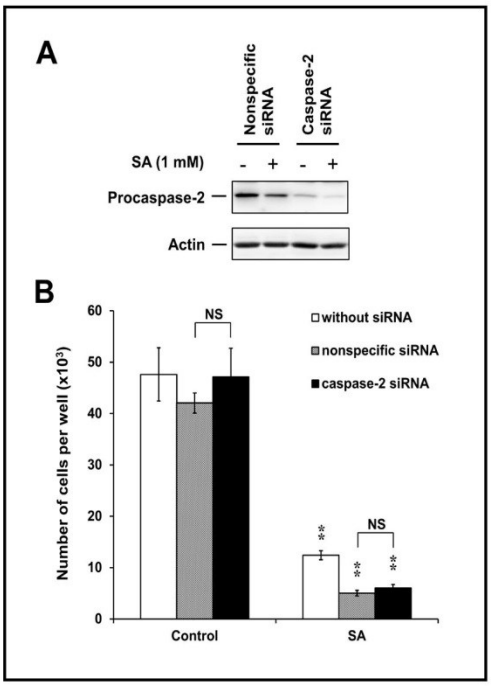


Fig. 6. Effect of caspase-2 silencing by specific siRNA on (A) caspase-2 expression and on (B) the effect of 1 mM stearic acid (SA) on the cell growth and viability of NES2Y cells. Cells incubated without stearic acid represented control cells. (A) The expression of caspase-2 after 24 h of incubation with stearic acid was determined by western blot analysis using caspase-2 specific antibody (see „Materials and Methods"). Monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment of three independent experiments. (B) Cells treated with nonspecific siRNA, caspase-2 specific siRNA and without siRNA as described in „Materials and Methods" were seeded at $2x10^4$ cells/100 µl of culture medium per well of 96-well plate and stearic acid was applied (see "Materials and Methods"). The number of living cells was determined after 48 h of incubation. Each column represents the mean of 4 separate cultures ± SEM. ** $P<0.01$ when comparing the effect of stearic acid with respective control cells, nonsignificant (NS) when comparing the effect of caspase-2 and nonspecific siRNA ($P \geq 0.05$).

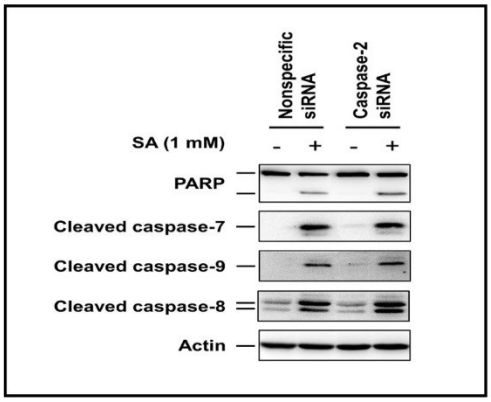


Fig. 7. Effect of caspase-2 silencing by specific siRNA on the effect of 1 mM stearic acid (SA) on the cleavage of PARP, caspase-7, caspase-9 and caspase-8 in NES2Y cells. Cells incubated without stearic acid represented control cells. After 18 h of incubation, the cleavage of proteins was determined by western blot analysis employing relevant antibodies (see „Materials and Methods"). Monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment of three independent experiments.

Effect of caspase-2 silencing on apoptosis induction and caspase activation

To assess the role of caspase-2 in apoptosis induced by stearic acid in NES2Y cells, we employed siRNA approach to silence caspase-2 expression. Using caspase-2 specific siRNA, we achieved 81±4% inhibition of caspase-2 expression in the latest time point tested, i.e. 24 h of treatment (Fig. 6A). First, we assessed the effect of caspase-2 silencing on the growth and viability of NES2Y cells during the treatment with stearic acid. After 48 h of treatment, there was no significant difference between the number of cells with down-regulated expression of caspase-2 and cells treated with control nonspecific siRNA. We also did not observe any significant effect of the transfection procedure per se on the growth and viability of NES2Y cells (Fig. 6B). Next, we tested the effect of inhibition of caspase-2 expression on PARP cleavage and the activation of caspase-7, -9 and -8. Activation of all caspases tested was readily detectable after 18 h of treatment, however, we did not see any inhibitory effect of caspase-2 silencing on the activation of caspases tested as well as on PARP cleavage (Fig. 7).

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013

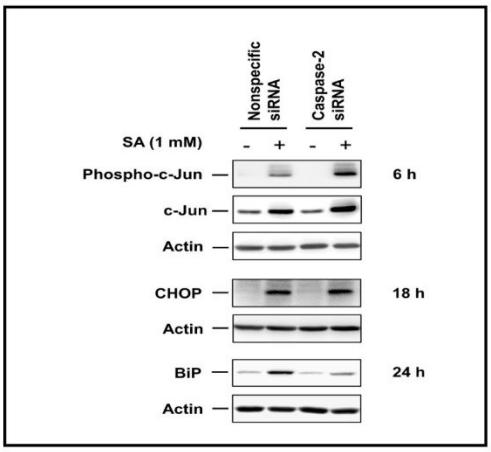


Fig. 8. Effect of caspase-2 silencing by specific siRNA on the effect of 1 mM stearic acid (SA) on the expression of phospho-c-Jun, c-Jun, CHOP and BiP in NES2Y cells. Cells incubated without stearic acid represented control cells. After 6 h (phospho-c-Jun and c-Jun), 18 h (CHOP) and 24 h (BiP) of incubation, the expression of proteins was determined by western blot analysis employing relevant antibodies (see „Materials and Methods"). Monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment of three independent experiments.

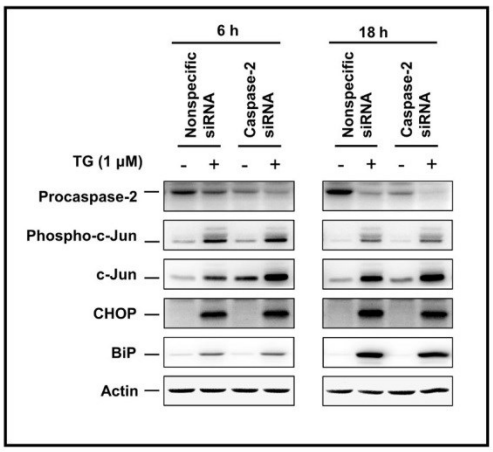


Fig. 9. Effect of caspase-2 silencing by specific siRNA on the effect of 1 μM thapsigargin (TG) on the expression of procaspase-2, phospho-c-Jun, c-Jun, CHOP and BiP in NES2Y cells. Cells incubated without thapsigargin represented control cells. After 6 h and 18 h of incubation, the expression of proteins was determined by western blot analysis employing relevant antibodies (see „Materials and Methods"). Monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment of three independent experiments.

Effect of caspase-2 silencing on ER stress

We did not detect any effect of caspase-2 silencing on the induction of CHOP expression after 18 and 24 h of stearic acid treatment (Fig. 8, only data for 18 h are shown). However, caspase-2 silencing led, similarly like the inhibition of JNK activity, to the decrease of BiP upregulation induced by stearic acid treatment for 18 h and 24 h (Fig. 8, only data for 24 h are shown). Surprisingly, we also found increased level of phosphorylated c-Jun in cells with silenced caspase-2 expression after 6 h, 18 h and 24 h of treatment with stearic acid (Fig. 8, only data for 6 h are shown). In order to compare the involvement of caspase-2 in the upregulation of BiP expression and c-Jun phosphorylation after stearic acid application (Fig. 8) with the involvement after ER stress induction, we tested the effect of caspase-2 silencing after ER stress induction by thapsigargin. However, we found no effect of caspase-2 silencing on thapsigargin-induced upregulation of BiP expression and c-Jun phoshorylation (Fig. 9).

Discussion

Our previous studies showed that saturated fatty acids (palmitic acid and, more efficiently, stearic acid) induce apoptosis associated with caspase-2 activation in human pancreatic β-cells NES2Y [8, 21]. These studies also pointed at a possible role of ER stress in apoptosis induction by saturated fatty acids [8]. Thus in this study we decided to test the role of caspase-2 and the ER stress mediator JNK in stearic acid-induced apoptosis of NES2Y cells considering their possible role in ER stress signaling.

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013

NES2Y cells respond to fatty acids similarly like primary human β-cells [2, 6, 7, 32], as we also found in our previous experiments [8, 21]. Thus it may be expected that the responses to saturated fatty acid seen in this study with NES2Y cells are comparable to the effects on human β-cells *in vivo*. Currently, there is a limited number of studies investigating the effect of fatty acids on ER stress signaling in relation to apoptosis in β-cells of human origin [6, 7, 32]. Moreover, the effect of stearate is studied only rarely despite it appears to be more effective than palmitate in human β-cells [1, 8].

We demonstrated activation of all known pathways of ER stress signaling, i.e. IRE1α, PERK and ATF6 pathway, by saturated stearic acid at a cell death-inducing concentration (1 mM) in NES2Y cells as soon as 3 h after the treatment. After that we detected upregulation of the expression of ER stress markers CHOP and BiP 6 h and 12 h after the treatment, respectively.

JNK is considered as one of the most possible mediators that connect excessive ER stress with apoptosis induction. However, in human NES2Y cells, inhibition of JNK activity did not lead to significant changes in the level of stearate-induced apoptosis (see Fig. 4C). In the study of Cunha et al. with rat β-cells INS-1E [6] inhibition of palmitate-induced JNK activation resulted in a partial reduction of apoptosis. In another study with INS-1E cells, JNK inhibition was showed to be anti-apoptotic [18]. In NES2Y cells, JNK activation by stearic acid was accompanied with intensive and fast c-Jun phosphorylation (see Fig. 1A). However, there are conflicting data concerning the question whether palmitate-induced JNK activation in INS-1E cells also leads to phosphorylation of c-Jun [18, 33]. Therefore it is somewhat difficult to compare the data. Nevertheless, none of the studies showed complete inhibition of fatty acid-induced apoptosis by JNK inhibition. It indicates that some other mediator(s) or pathway(s) different from JNK signaling should be involved in saturated fatty acid-induced apoptosis.

Several lines of evidence indicate that fatty acid-induced activation of JNK in β-cells is very fast and precedes the activation of ER stress response and therefore could not result solely from IRE1α activation [17, 19]. JNK can also be activated by MAP kinase kinases MKK4 and MKK7 and thus its activation by saturated fatty acids can result also from the involvement of signaling pathways that are not related to ER stress induction [16]. Our results do not contradict this hypothesis since we observed full JNK activation after 3 h but splicing of XBP1 reached its maximum at 6 h. To sum up, our data suggest that JNK signaling in NES2Y cells is not a mediator of stearic acid-induced apoptosis regardless of whether its activation is solely the result of ER stress induction or whether also other signaling pathways contribute to its activation.

In rodent cells, the activation of caspase cascade in response to ER stress is initiated by the activation of caspase-12 [34]. However, except of specific population of African descendents, the human population does not possess functional caspase-12 [35]. Several lines of evidence indicate that caspase-2 [22-25] or caspase-4 [36, 37] could substitute for caspase-12 in human cells but this hypothesis has not yet been tested in pancreatic β-cells. Our preliminary experiments did not point at a significant role of caspase-4 activation in stearic acid-induced apoptosis of NES2Y cells (data not shown). However, we previously identified that caspase-2 was significantly activated by palmitate and stearate in NES2Y cells [8, 21]. Caspase-2 was also shown to play the key role in GTP-depletion-induced β-cell apoptosis [38]. In this study we show not only that caspase-2 is dispensable for fatty acid-induced apoptosis in NES2Y but we also found no influence of caspase-2 silencing on the activation of other caspases previously shown to be associated with stearate-induced apoptosis [8].

During stearic acid-induced apoptosis in NES2Y cells, in contrast to JNK inhibition, caspase-2 silencing was accompanied by the increased phosphorylation of c-Jun. However, both caspase-2 silencing and JNK inhibition had similar effect on CHOP and BiP expression after stearic acid treatment (see Figs. 4A and 8). This indicates that CHOP and BiP expression in NES2Y cells are not regulated by c-Jun. This is consistent with the finding that dimers of transcription factor AP-1 that contribute to CHOP induction by palmitate in INS-1E β-cells are composed preferentially of c-Fos and jun-B, but not c-Jun [33] and the activity

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013

of jun-B is not controlled by JNK-mediated phosphorylation [39]. However, the mechanism mediating the decrease of BiP expression downstream of JNK inhibition (see Fig. 4A) and the mechanism responsible for increased c-Jun phosphorylation after caspase-2 silencing (see Fig. 8) remain obscure. Nevertheless, some regulatory role of JNK in BiP expression is likely, as demonstrated by the decrease of BiP expression by JNK inhibition during thapsigargin-induced ER stress (see Fig. 5) [40].

Downregulation of BiP expression due to caspase-2 silencing after stearic acid-induced apoptosis (see Fig. 8) is unexpected. As we did not observe this downregulation during thapsigargin-induced ER stress, the downregulation by caspase-2 silencing seems to be restricted to specific ER stress stimuli, such as saturated fatty acids. Caspase-2 was shown to be transcriptionally regulated by the sterol regulatory element binding protein 2 (SREBP2) and to participate together with this transcription factor in the activation of endogenous cholesterol and triacylglycerol synthesis [41, 42]. In this way, caspase-2 silencing might be associated with lowering of intracellular lipid levels which could be reflected in turn by the alleviation of ER stress and reduction in BiP expression induced by stearic acid.

Taken together, in this study we assessed the role of caspase-2 and JNK in stearic acid-induced apoptosis and ER stress in NES2Y cells. Stearic acid activates IRE1α, PERK and ATF6 pathways of ER stress signaling. Neither caspase-2 nor JNK activation is involved in apoptosis induction by saturated fatty acids. However, both caspase-2 and JNK modulate stearic acid-induced ER stress in NES2Y cells.

Acknowledgements

This work was supported by research program MSM0021620814 of the Third Faculty of Medicine, Charles University in Prague, provided by Ministry of Education, Youth and Sports of the Czech Republic and by research projects SVV-2010-260704, SVV-2011-262706, UNCE 204015 and PRVOUK P31 from the Charles University in Prague, Czech Republic. We would like to thank Dr. Lenka Rossmeislová for her technical assistance concerning confocal microscopy.

References

1 Eitel K, Staiger H, Brendel MD, Brandhorst D, Bretzel RG, Haring HU, Kellerer M: Different role of saturated and unsaturated fatty acids in beta-cell apoptosis. Biochem Biophys Res Commun 2002;299:853-856.
2 Maedler K, Oberholzer J, Bucher P, Spinas GA, Donath MY: Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. Diabetes 2003;52:726-733.
3 Diakogiannaki E, Welters HJ, Morgan NG: Differential regulation of the endoplasmic reticulum stress response in pancreatic beta-cells exposed to long-chain saturated and monounsaturated fatty acids. J Endocrinol 2008;197:553-563.
4 Harding HP, Zeng H, Zhang Y, Jungries R, Chung P, Plesken H, Sabatini DD, Ron D: Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival. Mol Cell 2001;7:1153-1163.
5 Kharroubi I, Ladriere L, Cardozo AK, Dogusan Z, Cnop M, Eizirik DL: Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress. Endocrinology 2004;145:5087-5096.
6 Cunha DA, Hekerman P, Ladriere L, Bazarra-Castro A, Ortis F, Wakeham MC, Moore F, Rasschaert J, Cardozo AK, Bellomo E, Overbergh L, Mathieu C, Lupi R, Hai T, Herchuelz A, Marchetti P, Rutter GA, Eizirik DL, Cnop M: Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. J Cell Sci 2008;121:2308-2318.

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013

7 Ladriere L, Igoillo-Esteve M, Cunha DA, Brion JP, Bugliani M, Marchetti P, Eizirik DL, Cnop M: Enhanced signaling downstream of ribonucleic Acid-activated protein kinase-like endoplasmic reticulum kinase potentiates lipotoxic endoplasmic reticulum stress in human islets. J Clin Endocrinol Metab 2010;95:1442-1449.
8 Nemcova-Furstova V, James RF, Kovar J: Inhibitory effect of unsaturated fatty acids on saturated fatty acid-induced apoptosis in human pancreatic beta-cells: activation of caspases and ER stress induction. Cell Physiol Biochem 2011;27:525-538.
9 Hartman MG, Lu D, Kim ML, Kociba GJ, Shukri T, Buteau J, Wang X, Frankel WL, Guttridge D, Prentki M, Grey ST, Ron D, Hai T: Role for activating transcription factor 3 in stress-induced beta-cell apoptosis. Mol Cell Biol 2004;24:5721-5732.
10 Huang CJ, Lin CY, Haataja L, Gurlo T, Butler AE, Rizza RA, Butler PC: High expression rates of human islet amyloid polypeptide induce endoplasmic reticulum stress mediated beta-cell apoptosis, a characteristic of humans with type 2 but not type 1 diabetes. Diabetes 2007;56:2016-2027.
11 Laybutt DR, Preston AM, Akerfeldt MC, Kench JG, Busch AK, Biankin AV, Biden TJ: Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. Diabetologia 2007;50:752-763.
12 Cnop M, Ladrière L, Igoillo-Esteve M, Moura RF, Cunha DA: Causes and cures for endoplasmic reticulum stress in lipotoxic β-cell dysfunction. Diabetes Obes Metab 2010;12:S76-82.
13 Fonseca SG, Gromada J, Urano F: Endoplasmic reticulum stress and pancreatic β-cell death. Trends Endocrinol Metab 2011;22:266-74.
14 Hetz C: The unfolded protein response: controlling cell fate decisions under ER stress and beyond. Nat Rev Mol Cell Biol 2012;13:89-102.
15 Tabas I, Ron D: Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. Nat Cell Biol 2011;13:184-190.
16 Dhanasekaran DN, Reddy EP: JNK signaling in apoptosis. Oncogene 2008;27:6245-6251.
17 Martinez SC, Tanabe K, Cras-Meneur C, Abumrad NA, Bernal-Mizrachi E, Permutt MA: Inhibition of Foxo1 protects pancreatic islet beta-cells against fatty acid and endoplasmic reticulum stress-induced apoptosis. Diabetes 2008;57:846-859.
18 Bachar E, Ariav Y, Ketzinel-Gilad M, Cerasi E, Kaiser N, Leibowitz G: Glucose amplifies fatty acid-induced endoplasmic reticulum stress in pancreatic beta-cells via activation of mTORC1. PLoS One 2009;4:e4954.
19 Tanabe K, Liu Y, Hasan SD, Martinez SC, Cras-Meneur C, Welling CM, Bernal-Mizrachi E, Tanizawa Y, Rhodes CJ, Zmuda E, Hai T, Abumrad NA, Permutt MA: Glucose and fatty acids synergize to promote B-cell apoptosis through activation of glycogen synthase kinase 3beta independent of JNK activation. PLoS One 2011;6:e18146.
20 Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, Tuncman G, Gorgun C, Glimcher LH, Hotamisligil GS: Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 2004;306:457-461.
21 Furstova V, Kopska T, James RF, Kovar J: Comparison of the effect of individual saturated and unsaturated fatty acids on cell growth and death induction in the human pancreatic beta-cell line NES2Y. Life Sci 2008;82:684-691.
22 Cheung HH, Lynn KN, Liston P, Korneluk RG: Involvement of caspase-2 and caspase-9 in endoplasmic reticulum stress-induced apoptosis: a role for the IAPs. Exp Cell Res 2006;312:2347-2357.
23 Gu H, Chen X, Gao G, Dong H: Caspase-2 functions upstream of mitochondria in endoplasmic reticulum stress-induced apoptosis by bortezomib in human myeloma cells. Mol Cancer Ther 2008;7:2298-2307.
24 Upton JP, Austgen K, Nishino M, Coakley KM, Hagen A, Han D, Papa FR, Oakes SA: Caspase-2 cleavage of BID is a critical apoptotic signal downstream of endoplasmic reticulum stress. Mol Cell Biol 2008;28:3943-3951.
25 Huang WC, Lin YS, Chen CL, Wang CY, Chiu WH, Lin CF: Glycogen synthase kinase-3beta mediates endoplasmic reticulum stress-induced lysosomal apoptosis in leukemia. J Pharmacol Exp Ther 2009;329:524-531.
26 Macfarlane WM, Cragg H, Docherty HM, Read ML, James RF, Aynsley-Green A, Docherty K: Impaired expression of transcription factor IUF1 in a pancreatic beta-cell line derived from a patient with persistent hyperinsulinaemic hypoglycaemia of infancy (nesidioblastosis). FEBS Lett 1997;413:304-308.

Cellular Physiology and Biochemistry
Cell Physiol Biochem 2013;31:277-289
DOI: 10.1159/000343367
Published online: February 28, 2013

27 Musilkova J, Kovar J: Additive stimulatory effect of extracellular calcium and potassium on non-transferrin ferric iron uptake by HeLa and K562 cells. Biochim Biophys Acta 2001;1514:117-126.
28 Kovar J, Franek F: Growth-stimulating effect of transferrin on a hybridoma cell line: relation to transferrin iron-transporting function. Exp Cell Res 1989;182:358-369.
29 Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG: Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. Diabetes 2001;50:1771-1777.
30 Yan Y, Gao YY, Liu BQ, Niu XF, Zhuang Y, Wang HQ: Resveratrol-induced cytotoxicity in human Burkitt's lymphoma cells is coupled to the unfolded protein response. BMC Cancer 2010;10:445.
31 Balusikova K, Neubauerova J, Dostalikova-Cimburova M, Horak J, Kovar J: Differing expression of genes involved in non-transferrin iron transport across plasma membrane in various cell types under iron deficiency and excess. Mol Cell Biochem 2009;321:123-133.
32 Lai E, Bikopoulos G, Wheeler MB, Rozakis-Adcock M, Volchuk A: Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic beta-cells. Am J Physiol Endocrinol Metab 2008;294:E540-E550.
33 Pirot P, Ortis F, Cnop M, Ma Y, Hendershot LM, Eizirik DL, Cardozo AK: Transcriptional regulation of the endoplasmic reticulum stress gene chop in pancreatic insulin-producing cells. Diabetes 2007;56:1069-1077.
34 Szegezdi E, Fitzgerald U, Samali A: Caspase-12 and ER-stress-mediated apoptosis: the story so far. Ann N Y Acad Sci 2003;1010:186-194.
35 Martinon F, Tschopp J: Inflammatory caspases and inflammasomes: master switches of inflammation. Cell Death Differ 2007;14:10-22.
36 Hitomi J, Katayama T, Eguchi Y, Kudo T, Taniguchi M, Koyama Y, Manabe T, Yamagishi S, Bando Y, Imaizumi K, Tsujimoto Y, Tohyama M: Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. J Cell Biol 2004;165:347-56.
37 Yamamuro A, Kishino T, Ohshima Y, Yoshioka Y, Kimura T, Kasai A, Maeda S: Caspase-4 directly activates caspase-9 in endoplasmic reticulum stress-induced apoptosis in SH-SY5Y cells. J Pharmacol Sci 2011;115:239-43.
38 Huo J, Luo RH, Metz SA, Li G: Activation of caspase-2 mediates the apoptosis induced by GTP-depletion in insulin-secreting (HIT-T15) cells. Endocrinology 2002;143:1695-1704.
39 Kallunki T, Deng T, Hibi M, Karin M: c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. Cell 1996;87:929-939.
40 Raciti M, Lotti LV, Valia S, Pulcinelli FM, Di Renzo L: JNK2 is activated during ER stress and promotes cell survival. Cell Death Dis 2012,3:e429.
41 Logette E, Le Jossic-Corcos C, Masson D, Solier S, Sequeira-Legrand A, Dugail I, Lemaire-Ewing S, Desoche L, Solary E, Corcos L: Caspase-2, a novel lipid sensor under the control of sterol regulatory element binding protein 2. Mol Cell Biol 2005;25:9621-9631.
42 Logette E, Solary E, Corcos L: Identification of a functional DNA binding site for the SREBP-1c transcription factor in the first intron of the human caspase-2 gene. Biochim Biophys Acta 2005;1738:1-5.

7.2. PUBLIKACE 2

Šrámek J, Němcová-Fürstová V, Balušíková K, Daniel P, Jelínek M, James RF, Kovář J: p38 MAPK is activated but does not play a key role during apoptosis induction by saturated fatty acid in human pancreatic β-cells.
International Journal of Molecular Sciences, 17: 159, 2016.

International Journal of
Molecular Sciences

MDPI

Article

p38 MAPK Is Activated but Does Not Play a Key Role during Apoptosis Induction by Saturated Fatty Acid in Human Pancreatic β-Cells

Jan Šrámek [1,*], Vlasta Němcová-Fürstová [1], Kamila Balušíková [1], Petr Daniel [1], Michael Jelínek [1], Roger F. James [2] and Jan Kovář [1,*]

1 Division of Cell and Molecular Biology & Center for Research of Diabetes, Metabolism and Nutrition, Third Faculty of Medicine, Charles University in Prague, Ruská 87, Prague 110 00, Czech Republic; vlasta.furstova@tiscali.cz (V.N.-F.); kamila.balusikova@lf3.cuni.cz (K.B.); petr85daniel@gmail.com (P.D.); michael.j@email.cz (M.J.)

2 Department of Infection, Immunity and Inflammation, Maurice Shock Medical Sciences Building, University of Leicester, University Road, Leicester LE1 9HN, UK; rj1@leicester.ac.uk

* Correspondence: jan.sramek@seznam.cz (J.S.); jan.kovar@lf3.cuni.cz (J.K.); Tel.: +420-267-102-658 (J.S. & J.K.)

Academic Editor: Charles J. Malemud
Received: 2 December 2015; Accepted: 22 January 2016; Published: 5 February 2016

Abstract: Saturated stearic acid (SA) induces apoptosis in the human pancreatic β-cells NES2Y. However, the molecular mechanisms involved are unclear. We showed that apoptosis-inducing concentrations of SA activate the p38 MAPK signaling pathway in these cells. Therefore, we tested the role of p38 MAPK signaling pathway activation in apoptosis induction by SA in NES2Y cells. Crosstalk between p38 MAPK pathway activation and accompanying ERK pathway inhibition after SA application was also tested. The inhibition of p38 MAPK expression by siRNA silencing resulted in a decrease in MAPKAPK-2 activation after SA application, but it had no significant effect on cell viability or the level of phosphorylated ERK pathway members. The inhibition of p38 MAPK activity by the specific inhibitor SB202190 resulted in inhibition of MAPKAPK-2 activation and noticeable activation of ERK pathway members after SA treatment but in no significant effect on cell viability. p38 MAPK overexpression by plasmid transfection produced an increase in MAPKAPK-2 activation after SA exposure but no significant influence on cell viability or ERK pathway activation. The activation of p38 MAPK by the specific activator anisomycin resulted in significant activation of MAPKAPK-2. Concerning the effect on cell viability, application of the activator led to apoptosis induction similar to application of SA (PARP cleavage and caspase-7, -8, and -9 activation) and in inhibition of ERK pathway members. We demonstrated that apoptosis-inducing concentrations of SA activate the p38 MAPK signaling pathway and that this activation could be involved in apoptosis induction by SA in the human pancreatic β-cells NES2Y. However, this involvement does not seem to play a key role. Crosstalk between p38 MAPK pathway activation and ERK pathway inhibition in NES2Y cells seems likely. Thus, the ERK pathway inhibition by p38 MAPK activation does not also seem to be essential for SA-induced apoptosis.

Keywords: p38 MAPK; ERK; fatty acids; pancreatic β-cells; apoptosis; NES2Y

1. Introduction

Increased levels of fatty acids (FAs) in blood are considered to be one of the main factors responsible for pancreatic β-cell death in type 2 diabetes [1–5]. Our previous studies as well as others have shown that saturated FAs induce apoptosis in pancreatic β-cells [2,4–8]. Although the precise molecular mechanisms of apoptosis induction by FAs in β-cells remain unclear [9], there are

some indications that the p38 MAPK (mitogen-activated protein kinase) signaling pathway could be involved [10,11].

p38 MAPK kinase has been shown to participate in the regulation of many cellular processes such as cell proliferation, differentiation, the inflammatory response, and apoptosis (reviewed in [12]). Generally, p38 MAPK is activated via the dual-specific MKK3/6 (mitogen-activated protein kinase kinase 3 and 6), kinase in response to various extracellular stimuli such as physical and chemical stress (reviewed in [12]). p38 MAPK regulates activation of MAPKAPK-2 (MAPK-activated protein kinase 2), which is responsible for nuclear export of activated p38 MAPK [13] and can also affect activation of certain proteins involved in apoptosis regulation such as NF-κB (nuclear factor κB) [14] or caspase-3 [15]. Concerning apoptosis, it has been demonstrated, in different cell types and under different experimental conditions, that p38 MAPK can mediate pro-apoptotic signaling [16,17]. The pro-apoptotic function of p38 MAPK has also been demonstrated in studies using pancreatic β-cells exposed to FAs. However, the possible role of p38 MAPK in FA-induced apoptosis in pancreatic β-cells remains unclear [10,18,19].

Several studies [16,20] have shown that in some types of cells, p38 MAPK can inhibit the c-Raf → MEK1/2 → ERK1/2 (extracellular signal-regulated kinases 1 and 2) pathway, *i.e.*, the ERK signaling pathway. The ERK pathway is mostly activated by growth factors. These factors, acting through receptor kinases and adaptor protein son of sevenless (SOS), activate Ras GTPase, which is responsible for c-Raf phosphorylation [21]. Like p38 MAPK, the ERK pathway can affect various proteins associated with apoptosis, e.g., Fox03a or several proteins of the Bcl-2 family [22]. It has been demonstrated that the ERK pathway can mediate both pro-apoptotic as well as anti-apoptotic signaling [23–26]. Regulation of ERK1/2 activation by saturated FAs in pancreatic β-cells has also been shown [27,28].

There are data supporting the idea that the p38 MAPK signaling pathway could be involved in apoptosis induction by FAs in pancreatic β-cells [10,18]. In the present study, we demonstrated that p38 MAPK is activated during apoptosis induction by stearic acid (SA) in the human pancreatic β-cells NES2Y. Therefore, we tested the role of p38 MAPK signaling pathway activation in apoptosis induction by SA, representing saturated FAs, in NES2Y cells. Crosstalk between p38 MAPK pathway activation and ERK pathway inhibition, after SA application, was also tested. We demonstrated that the activation of the p38 MAPK pathway could be somehow involved in apoptosis induction by SA in the human pancreatic β-cells NES2Y. However, this involvement does not seem to play a key role. Crosstalk between p38 MAPK pathway activation and ERK pathway inhibition in NES2Y cells seems likely. Thus, the ERK pathway inhibition by p38 MAPK activation does not also seem to be essential for SA-induced apoptosis.

2. Results

2.1. Effect of Stearic Acid on Cell Death Induction and Activation of Members of the p38 MAPK and ERK Signaling Pathways

We assessed cell viability as well as cleavage of PARP (a common marker of apoptosis) and caspase-7, -8 and -9 activation by cleavage after SA (1 mM) exposure, in NES2Y cells. SA application resulted in significant induction of cell death within 48 h after SA application (Figure 1A), and increased caspase-7, -8, -9 and PARP (a substrate of activated executioner caspases) cleavage 18 h after SA application (Figure 1B). Previously, it was found that there is nearly no activation of caspase-3 in NES2Y cells after SA exposure, and that caspase-2 does not play a key role in SA-induced apoptosis [8,29].

Next, we assessed the levels of activated (phosphorylated) members of the p38 MAPK signaling pathway (phospho-MKK3/6, phospho-p38 MAPK, phospho-MAPKAPK-2) as well as the levels of activated members of the ERK signaling pathway (phospho-c-Raf, phospho-MEK1/2, phospho-ERK1/2) within 24 h after SA application, in NES2Y cells.

SA treatment resulted in an increase in the level of phosphorylated members of the p38 MAPK pathway as early as 3 h after application. The level of phosphorylation increased to a maximum at 12 h

Int. J. Mol. Sci. **2016**, *17*, 159

after application for all tested proteins. At 24 h after treatment, the level of phosphorylation decreased. No change was detected in the level of total p38 MAPK during 24 h after SA application (Figure 1C).

Levels of phosphorylated members of the ERK pathway decreased as early as 3 h after SA application, except for MEK1/2. The effect of SA increased to the maximum for all tested proteins 12–24 h after application. We did not detect any change in the level of total ERK1/2 during 24 h after SA application (Figure 1D).

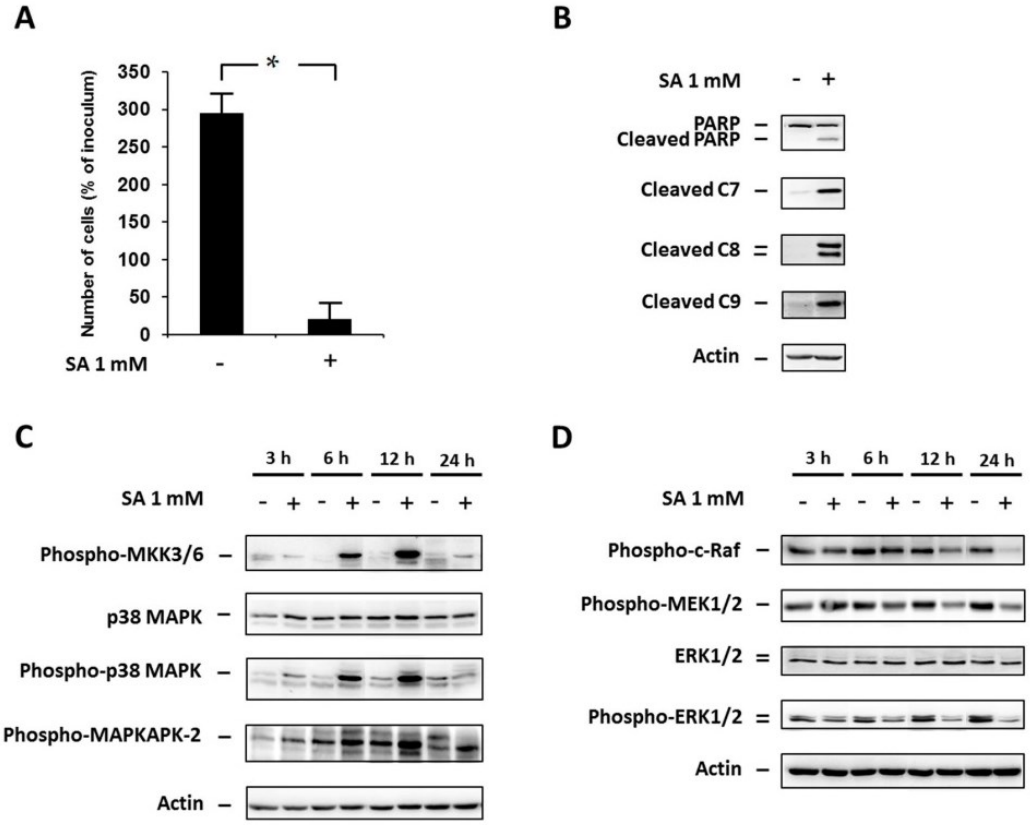


Figure 1. Effect of 1 mM stearic acid (SA) (see "Materials and Methods") on (**A**) cell growth and viability; (**B**) the level of cleaved PARP, caspase-7 (C7), caspase-8 (C8) and caspase-9 (C9) (markers of apoptosis); (**C**) the level of phospho-MKK3/6, p38 MAPK, phospho-p38 MAPK, phospho-MAPKAPK-2 (p38 MAPK signaling pathway); and (**D**) the level of phospho-c-Raf, phospho-MEK1/2, ERK1/2, phospho-ERK1/2 (the ERK signaling pathway) in NES2Y cells. Cells incubated without SA represented control cells. After 18 h of incubation (see "Materials and Methods") for markers of apoptosis (**B**) and 3, 6, 12 and 24 h of incubation for p38 MAPK and ERK pathways members (**C**,**D**), the levels of individual proteins were determined using Western blot analysis and the relevant antibodies (see "Materials and Methods"). Monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment from at least three independent experiments. When assessing cell growth and viability (**A**), cells were seeded at a concentration of 2×10^4 cells/100 μL of culture medium per well of 96-well plate (see "Materials and Methods"). The number of living cells was determined after 48 h of incubation. Each column represents the mean of four separate cultures ± standard error of the mean (SEM). * $p < 0.05$ when comparing the number of control cells and cells treated with SA.

2.2. *Effect of p38 MAPK Silencing*

In order to test the involvement of p38 MAPK in apoptosis signaling induced by SA in NES2Y cells, we assessed the effect of p38 MAPK silencing by specific siRNA on cell growth and viability after

Int. J. Mol. Sci. **2016**, *17*, 159

SA treatment. We also tested the effect of p38 MAPK silencing on phosphorylation of MAPKAPK-2 (pathway member downstream of p38 MAPK) and phosphorylation of ERK pathway members (c-Raf, MEK1/2 and ERK1/2) after SA application. To assess the efficiency of silencing, we measured the level of total p38 MAPK and phospho-p38 MAPK, respectively.

p38 MAPK silencing (approximately 60%) resulted in a decrease in phospho-p38 MAPK level, which was expected, and also a decrease in phospho-MAPKAPK-2 level 18 h after SA application (Figure 2A). However, it had nearly no effect on the level of phosphorylated ERK pathway members (Figure 2B). Cell viability was not significantly affected by p38 MAPK silencing during 48 h after SA treatment (Figure 2C)

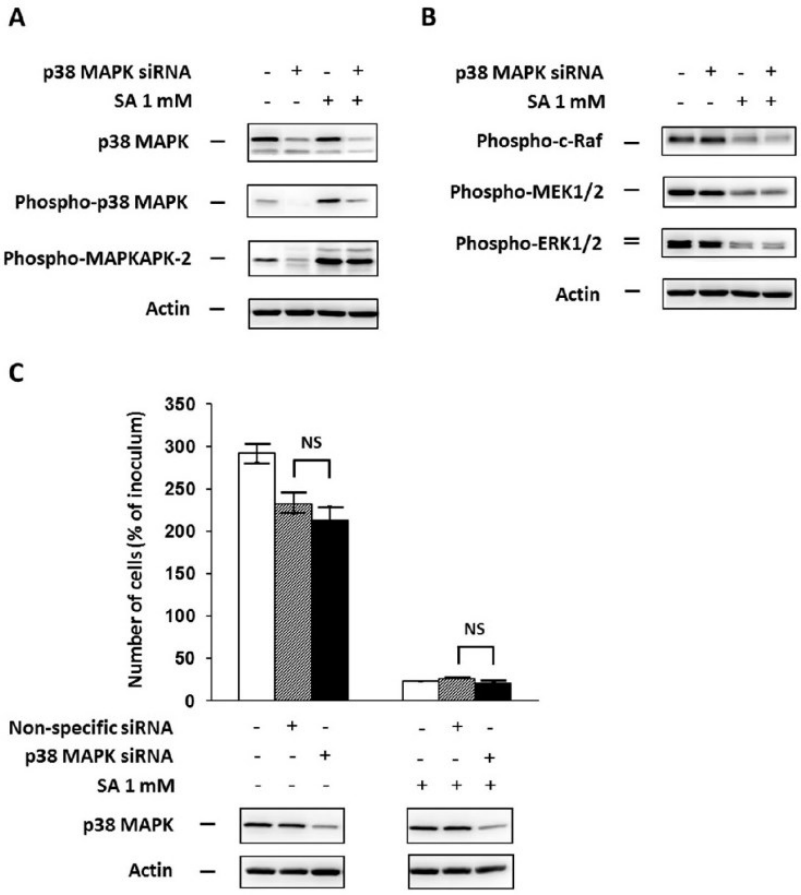


Figure 2. Effect of p38 MAPK silencing, using a specific siRNA (see "Materials and Methods") and the effect of stearic acid (SA), on (**A**) the level of p38 MAPK, phospho-p38 MAPK, phospho-MAPKAPK-2 (substrate of p38 MAPK); (**B**) the level of phospho-c-Raf, phospho-MEK1/2, phospho-ERK1/2 (the ERK signaling pathway); and (**C**) cell growth and viability of NES2Y cells. Cells incubated without siRNA represented control cells. After 18 h of incubation (see "Materials and Methods") with or without stearic acid (SA) (**A**,**B**), the level of individual proteins was determined using Western blot analysis and the relevant antibodies (see "Materials and Methods"). A monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment from three independent experiments. When assessing cell growth and viability (**C**), cells were seeded at a concentration of 2×10^4 cells/100 µL of culture medium per well of 96-well plate (see "Materials and Methods"). The number of living cells was determined after 48 h of incubation with or without SA. Non-specific siRNA was used as a negative control. Each column represents the mean of four separate cultures ± SEM. NS (non-significant) when comparing the number of cells incubated with p38 MAPK specific siRNA and with non-specific siRNA.

Int. J. Mol. Sci. **2016**, *17*, 159

2.3. Effect of the Specific p38 MAPK Inhibitor SB202190

We also assessed the effect of inhibition of p38 MAPK activity, using the specific inhibitor SB202190, on cell growth and viability, phosphorylation of MAPKAPK-2 (pathway member downstream of p38 MAPK), and phosphorylation of ERK pathway members (c-Raf, MEK1/2 and ERK1/2) after SA treatment in NES2Y β-cells. To assess the efficiency of inhibition, we measured the level of phospho-MAPKAPK-2.

p38 MAPK inhibition resulted in a decrease in phospho-MAPKAPK-2 level (Figure 3A) and in an increase of the levels of phosphorylated ERK pathway members (Figure 3B) 12 h after SA application. p38 MAPK inhibition in cells without SA exposure also increased the levels of phosphorylated ERK pathway members (Figure 3B). Cell viability was not significantly affected by the p38 MAPK inhibition within the 48-h period after SA treatment (Figure 3C).

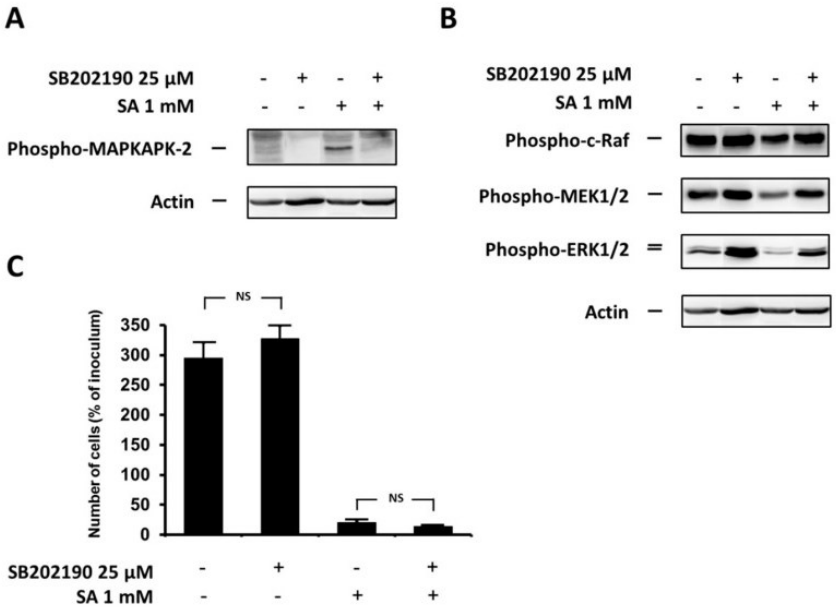


Figure 3. Effect of the specific p38 MAPK inhibitor, SB202190, (see "Materials and Methods") and the effect of stearic acid (SA) on (**A**) the level of phospho-MAPKAPK-2 (substrate of p38 MAPK); (**B**) the level of phospho-c-Raf, phospho-MEK1/2, phospho-ERK1/2 (the ERK signaling pathway); and (**C**) cell growth and viability of NES2Y cells. Cells incubated without the inhibitor represented control cells. After 12 h of incubation (see "Materials and Methods") (**A**,**B**), the level of individual proteins was determined using Western blot analysis and the relevant antibodies (see "Materials and Methods"). A monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment from three independent experiments. When assessing cell growth and viability (**C**), cells were seeded at a concentration of 2×10^4 cells/100 µL of culture medium per well of 96-well plate (see "Materials and Methods"). The number of living cells was determined after 48 h of incubation. Each column represents the mean of four separate cultures ± SEM. NS (non-significant) when comparing the number of control cells and cells treated with SB202190 as well as when comparing the effect of SA alone and applied together with SB202190.

2.4. Effect of p38 MAPK Overexpression

Next, we assessed the effect of p38 MAPK overexpression, through specific plasmid transfection, on cell growth and viability, phosphorylation of MAPKAPK-2 (pathway member downstream of p38 MAPK), and phosphorylation of ERK pathway members (c-Raf, MEK1/2 and ERK1/2) after SA

Int. J. Mol. Sci. **2016**, *17*, 159

exposure in NES2Y β-cells. To assess the efficiency of overexpression, we measured the level of total p38 MAPK and phospho-p38 MAPK, respectively.

Significant p38 MAPK overexpression resulted in an expected increase in phospho-p38 MAPK level and also an increase in phospho-MAPKAPK-2 level 18 h after SA application (Figure 4A). However, it had no effect on the level of phosphorylated ERK pathway members (Figure 4B). Cell viability was not significantly affected by p38 MAPK overexpression during 48 h after SA treatment (Figure 4C).

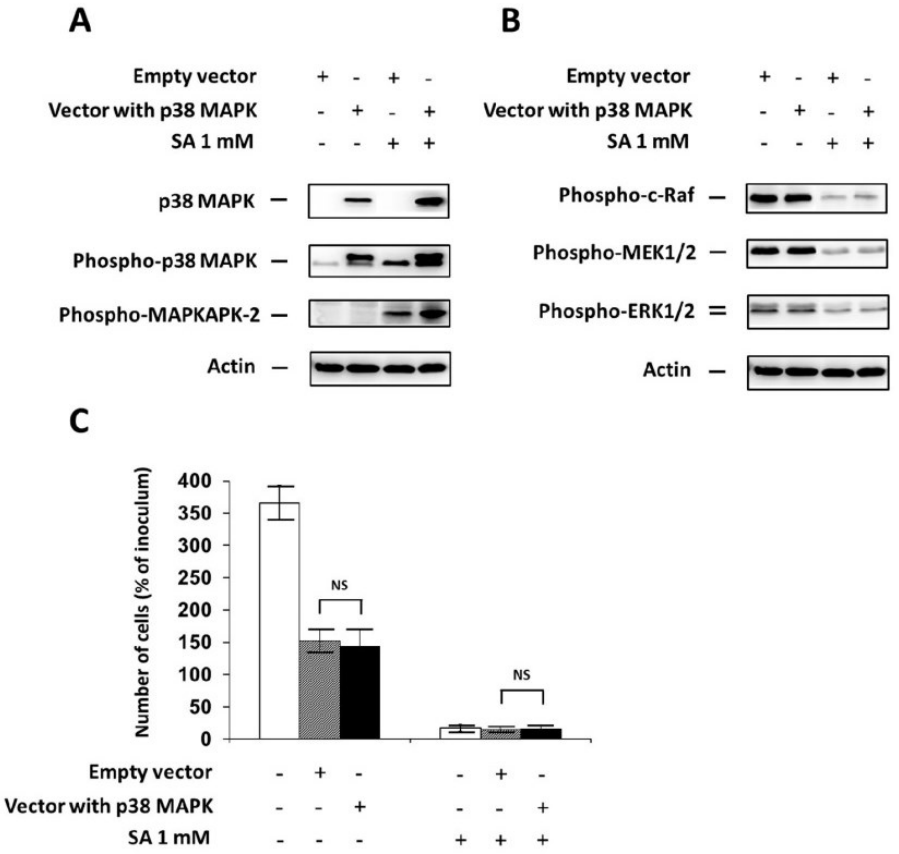


Figure 4. Effect of p38 MAPK overexpression, using transfection with a specific plasmid (Vector with p38 MAPK) (see "Materials and Methods") and the effect of stearic acid (SA) on (**A**) the level of p38 MAPK, phospho-p38 MAPK and phospho-MAPKAPK-2 (substrate of p38 MAPK); (**B**) the level of phospho-c-Raf, phospho-MEK1/2, phospho-ERK1/2 (the ERK signaling pathway); and (**C**) cell growth and viability of NES2Y cells. Cells transfected with an empty vector (Empty vector) represented control cells. After 18 h of incubation (see "Materials and Methods") with or without stearic acid (SA) (**A**,**B**), the level of individual proteins was determined using Western blot analysis and the relevant antibodies (see "Materials and Methods"). A monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment from three independent experiments. The fact that the band of p38 MAPK in the control samples is not visible here resulted from a large difference in p38 MAPK content in control and transfected cells. When assessing cell growth and viability (**C**), cells were seeded at a concentration of 2×10^4 cells/100 μL of culture medium per well of 96-well plate (see "Materials and Methods"). The number of living cells was determined after 48 h of incubation with or without SA. Each column represents the mean of four separate cultures ± SEM. NS (non-significant) when comparing the number of cells incubated with plasmid DNA containing p38 MAPK (Vector with p38 MAPK) and cells incubated with empty plasmid DNA (empty vector).

Int. J. Mol. Sci. **2016**, *17*, 159

2.5. Effect of the Specific p38 MAPK Activator Anisomycin

Lastly, we also assessed the effect of p38 MAPK activation, using the specific activator anisomycin, on cell growth and viability, phosphorylation of MAPKAPK-2 (pathway member downstream of p38 MAPK), and phosphorylation of ERK pathway members (c-Raf, MEK1/2 and ERK1/2) in NES2Y β-cells. To assess the efficiency of activation, we measured the level of phospho-p38 MAPK as well as the level of phospho-MAPKAPK-2.

Anisomycin-induced p38 MAPK activation resulted in a strong activation (phosphorylation) of MAPKAPK-2 (Figure 5A), 12 h after treatment. This activation appeared to be stronger than SA-induced activation. Anisomycin-induced p38 MAPK activation also led to a decrease in levels of the phosphorylated ERK pathway members, similar to that seen in SA-treated cells (Figure 5B). Furthermore, p38 MAPK activation resulted in induction of cell death (Figure 5C) within 48 h after anisomycin application, again similar to that seen in SA-treated cells.

Because cell death was induced, we also tested the effect of p38 MAPK activation on the cleavage of PARP (a common marker of apoptosis) and caspase-7, -8 and -9 activation. Caspase-7, -8, -9 activation as well as PARP cleavage was detected 12 h after activator application (Figure 5D).

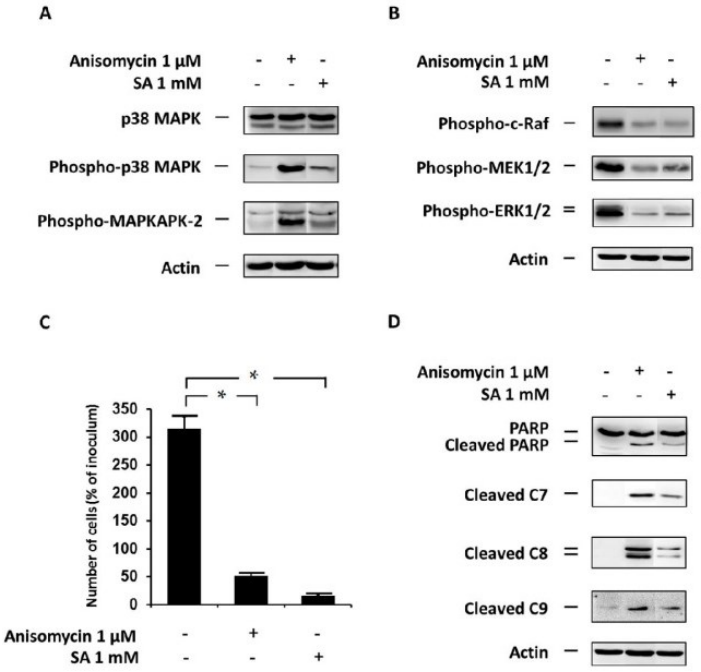


Figure 5. Effect of the specific p38 MAPK activator, anisomycin, (see "Materials and Methods") and the effect of stearic acid (SA) on (**A**) the level of p38 MAPK, phospho-p38 MAPK and phospho-MAPKAPK-2 (substrate of p38 MAPK); (**B**) the level of phospho-c-Raf, phospho-MEK1/2, phospho-ERK1/2 (the ERK signaling pathway); (**C**) cell growth and viability; and (**D**) cleavage of PARP, caspase-7 (C7), caspase-8 (C8) and caspase-9 (C9) (markers of apoptosis) in NES2Y cells. Cells incubated without the activator and SA represented control cells. After 12 h of incubation (see "Materials and Methods") (**A**,**B**,**D**), the level of individual proteins was determined using western blot analysis and the relevant antibodies (see "Materials and Methods"). A monoclonal antibody against human actin was used to confirm equal protein loading. The data shown were obtained in one representative experiment from three independent experiments. When assessing cell growth and viability (**C**), cells were seeded at a concentration of 2×10^4 cells/100 µL of culture medium per well of 96-well plate (see "Materials and Methods"). The number of living cells was determined after 48 h of incubation. Each column represents the mean of four separate cultures ± SEM. * $p < 0.05$ when comparing the number of control cells and cells with anisomycin or SA.

3. Discussion

Our previous papers as well as this study demonstrate that saturated fatty acids (palmitic and stearic acid) induced apoptosis in the human pancreatic β-cell line NES2Y (see Figure 1A,B) [5,8,29]. Molecular mechanisms of apoptosis induction by saturated fatty acids (FAs) in β-cells have not been fully elucidated yet [9]. Some studies indicated a possible involvement of various kinases in pro-apoptotic signaling (e.g., [10,27,30]). In this study, we tested the involvement of the p38 MAPK signaling pathway in stearic acid (SA)-induced apoptosis in the human pancreatic β-cells NES2Y. Furthermore, this study showed that, together with p38 MAPK pathway activation, apoptosis-inducing SA also inhibits the ERK pathway in NES2Y cells. Thus, we also tested crosstalk between p38 MAPK pathway activation and ERK pathway inhibition after SA application. To our knowledge, there is no detailed study considering deeply the effect of saturated FAs on p38 MAPK signaling pathway in relation to apoptosis induction in β-cells of human origin. Moreover, the effect of stearate is studied only rarely despite it appears to be more effective than palmitate in human β-cells [8,31]. Human NES2Y cells respond to FAs similarly like primary human β-cells [2,32–34], as we also found in our previous experiments [5,8]. Thus, it may be expected that the responses to saturated SA seen in this study with NES2Y cells could be more or less relevant to its effects on human β-cells *in vivo*.

As mentioned above, we demonstrated that apoptosis-inducing SA activates the p38 MAPK pathway in NES2Y β-cells (see Figure 1C). Activation of p38 MAPK in response to palmitic acid has already been documented in NIT-1 and RIN pancreatic β-cells [10,18]. Some studies [35,36] as well as our preliminary data indicate that SA-induced pro-apoptotic signaling could begin on the plasma membrane of cells probably due to SA effects on membrane fluidity. It is likely that it happens upstream of p38 MAPK signaling. The effect of SA on membrane fluidity can result in changes in the activity of some membrane receptor(s) or membrane-associated protein(s), which can mediate further signaling.

We also demonstrated that apoptosis-inducing SA inhibits the ERK pathway in NES2Y β-cells (see Figure 1D). Inhibition of ERK1/2 activity, in response to palmitic acid, has already been documented in some studies with pancreatic β-cell lines [27,28]. However, in other papers, activation of ERK1/2 was shown [37,38]. Moreover, the experimental conditions used in these studies have been quite dissimilar. As with the effect of SA on p38 MAPK activation, the effect of SA probably starts upstream of the ERK pathway. Some papers [16,20] suggest that a possible regulator of this pathway might be p38 MAPK kinase. In this paper, we showed that p38 MAPK kinase was activated by SA (see above).

Inhibition of p38 MAPK expression by siRNA silencing and inhibition of p38 MAPK activity by the specific inhibitor SB202190 after SA application, as well as p38 MAPK overexpression using plasmid transfection, had no significant effect on cell viability (see Figures 2C, 3C and 4C). However, the application of the specific p38 MAPK activator, anisomycin, resulted in apoptosis induction similar to that seen after application of SA (see Figure 5C). It also resulted in PARP cleavage and caspase-7, -8, -9 activation (see Figure 5D).

The insignificant effect of p38 MAPK silencing on cell viability after SA treatment could have resulted from incomplete inhibition of p38 MAPK expression (see Figure 2A). The remaining amount of p38 MAPK was still able to transduce a sufficient signal for apoptosis induction. The second possibility of explanation is that the p38 MAPK signaling pathway does not play a key role in apoptosis induction after SA treatment. The insignificant effect of the inhibitor application on cell viability could be explained by a similar way like the insignificant effect of p38 MAPK silencing. The insignificant effect of p38 MAPK overexpression, accompanied by an increased level of phospho-p38 MAPK (see Figure 4A), rather supports the possibility that the p38 MAPK pathway does not play a key role.

Concerning the effect of the activator, it should be mentioned that anisomycin also has the potential to affect, besides p38 MAPK, the activation of other molecules. Thus, the described effect of the activator on cell viability may not be mediated by p38 MAPK. Nonetheless, there is a possibility that the p38 MAPK pathway is somehow involved in SA-induced pro-apoptotic signaling in NES2Y cells. However, it probably does not represent the main pathway of the pro-apoptotic SA effect. The

possibility of a pro-apoptotic role for p38 MAPK has also been mentioned in other types of pancreatic β-cells or islets exposed to saturated FAs [10,18,39]. Nevertheless, in these cells, p38 MAPK seems to be more important as a mediator of saturated FA-induced apoptosis.

We can just speculate as to possible mechanisms playing a substantial role in saturated FA-induced apoptosis in pancreatic β-cells. These mechanisms probably represent a network of multiple signaling pathways. It is clear that caspases are involved here [8,32,40,41]. Generally, the involvement of the mitochondrial as well as the receptor pathways of apoptosis induction was documented in pancreatic β-cell lines or islets of animal or human origin (e.g., [2,40,42–45]). The involvement of inducible forms of nitric oxide synthase activation and nitric oxide production seems also speculative [1,6,46,47]. Experimental evidence strongly supports the role of *de novo* ceramide formation in saturated FA-induced apoptosis [2,42,46]. Other considered mechanisms that might play a role in regulation of β-cell viability by saturated FAs are activation of protein kinase Cδ [30], degradation of carboxypeptidase E [48], calpain-10 activation [41], activation of the transcription factor NF-κB [49,50], inhibition of protein kinase B [40], and the level of stearoyl-CoA desaturase-1 expression [33,51]. Nevertheless, the most studied molecular mechanism suggested to mediate FA-induced apoptosis is signaling of endoplasmic reticulum stress [7,29,32,34,49,52,53].

p38 MAPK silencing had no significant effect on ERK pathway activation (see Figure 2B). This could again be the result of incomplete inhibition of p38 MAPK expression (see above). On the other hand, application of the p38 MAPK inhibitor, SB202190, resulted in recognizable activation of ERK pathway members (see Figure 3B). However, it should be mentioned that this inhibitor effect could be the result of a direct effect of the inhibitor on the ERK pathway, since activation of c-Raf by SB202190 has been documented [54].

No significant effect on ERK pathway activation was also detected after p38 MAPK overexpression (see Figure 4B) while the application of p38 MAPK activator, anisomycin, resulted in significant inhibition of activation of ERK pathway members (see Figure 5B). Unfortunately, no significant effect of p38 MAPK overexpression, accompanied by increased level of phospho-p38 MAPK (see Figure 4A), on ERK pathway activation does not support the possibility of crosstalk. Regarding the effect of the activator, it should be noted that the effect of anisomycin on ERK pathway activation might not necessarily be mediated by p38 MAPK, since the activator can also affect other molecules. Although some of the approaches used to regulate p38 MAPK activation had no significant effect on ERK pathway activation; it seems that p38 MAPK kinase activation has an inhibitory effect on the ERK pathway in NES2Y β-cells after SA application. To date, no data documenting possible crosstalk between the p38 MAPK pathway and the ERK pathway, in pancreatic β-cells, has been published.

Taken together, we demonstrated that SA at apoptosis-inducing concentrations activates the p38 MAPK signaling pathway in human β-cells. We suggest that the activation of the p38 MAPK signaling pathway could be somehow involved in apoptosis induction by SA. However, this involvement does not seem to play a key role. Crosstalk between p38 MAPK pathway activation and the accompanying inhibition of the ERK signaling pathway after SA application seems more likely. Thus, the ERK pathway inhibition by p38 MAPK activation does not also seem to be essential for SA-induced apoptosis in human β-cells.

4. Materials and Methods

4.1. Materials

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. For western blot analysis, the following primary and secondary antibodies were used: anti-phospho-MKK3/6 (#9236), anti-p38 MAPK (#8690), anti-phospho-p38 MAPK (#4511), anti-phospho-MAPKAPK-2 (#3007), anti-phospho-c-Raf (#9427), anti-phospho-MEK1/2 (#9154), anti-ERK1/2 (#5013), anti-phospho-ERK1/2 (#4370), anti-PARP (#9542), anti-cleaved caspase-7 (#9491),

anti-cleaved caspase-8 (#9496), anti-cleaved caspase-9 (#9505) from Cell Signaling Technology (Danvers, MA, USA) and anti-actin (clone AC-40).

4.2. Cells and Culture Conditions

The human pancreatic β-cell line NES2Y [5,55] was used. NES2Y cells are proliferating insulin-secreting cells with a defect in glucose responsiveness. Cells were routinely maintained in an RPMI 1640 based culture medium [56]. In experiments, a defined serum-free medium [57] supplemented with 1 mM SA bound to 2% FA-free bovine serum albumin (BSA) was used [5]. Stock solutions containing SA bound to 10% BSA in a serum-free medium were prepared as described previously [5] and diluted to the required concentration of SA and BSA prior to experiments. SA/BSA molar ratios used in experiments were lower than the ratios known to exceed the binding capacity of BSA [58].

Our previous studies showed that SA, at a concentration of 1 mM, induced cell death in most NES2Y cells within 48 h of application. Apoptotic cells appeared within 24 h after SA application [5,8,29]. Therefore, all assessments were performed within 24 h after SA application, except for the assessment of cell growth and viability.

4.3. Assessment of the Effect of Stearic Acid on Cell Growth and Viability

Cells were seeded at a concentration of 2×10^4 cells/100 µL of culture media into the wells of 96-well plate. After a 24-h pre-incubation period (allowing cells to attach) the culture medium was replaced with a serum-free medium containing 2% BSA with or without SA. The control medium contained 2% BSA only. After 48 h of incubation, the number of living cells was determined using a hemocytometer counting system, after staining with trypan blue.

4.4. Western Blot Analysis

Cells (approximately 1×10^6 cells per sample) were seeded and after a 24-h pre-incubation period (allowing cells to attach), the culture medium was replaced with a serum-free medium containing 2% BSA with or without SA. The control medium contained 2% BSA only. After the required incubation period, cells were harvested and Western blot analysis was performed as described previously [8]. All primary antibodies were used in a 1:1000 dilution. The chemiluminescent signal was detected using a Carestream Gel Logic 4000 PRO Imaging System equipped with Carestream Molecular Imaging Software (Carestream Health, New Haven, CT, USA), which was used for image acquisition.

4.5. p38 MAPK Silencing by siRNA Transfection

To silence p38 MAPK expression, p38 MAPK specific siRNA (s3586, Life Technologies, Carlsbad, CA, USA) and INTERFERin (PolyPlus-Transfection, Illkirch, France), as transfection reagent, were used according to the manufacturer's instructions.

To silence p38 MAPK, 2.1×10^5 cells/6 mL were seeded into Petri dishes (Ø 6 cm). After 24 h (allowing cells to attach), the culture medium was replaced with new culture medium with or without the p38 MAPK specific siRNA and transfection reagent. The siRNA and transfection reagent were diluted in Opti-MEM® Reduced Serum Medium (Life Technologies) to a final concentration of 150 nM and 0.4%, respectively, prior to transfection. After 72 h of incubation, cells were harvested and seeded into six-well plates at a density of 1×10^6 cells/2.5 mL per well. After 24 h (allowing cells to attach), the culture medium was replaced with a serum-free medium containing: (1) fresh siRNA, transfection reagent (at the same concentration used for the initial inhibition of p38 MAPK expression) and 2% BSA with or without SA; or (2) 2% BSA with or without SA (control media). After 18 h of treatment, cells were harvested and lysates were prepared for western blot analysis as described previously [8]. The efficiency of p38 MAPK expression silencing was tested in each experiment at the protein level using Western blot analysis.

4.6. Assessment of the Effect of p38 MAPK Silencing on Cell Growth and Viability

The experiment was set up in the same way as described in "p38 MAPK silencing by siRNA transfection" with the following modifications. After 72 h of incubation with or without the specific or non-specific siRNA and transfection reagent, cells were seeded at a concentration of 2×10^4 cells/100 µL of relevant medium (see above) into the wells of 96-well plate. After 48 h of incubation with or without SA, the number of living cells was determined using a hemocytometer counting system after staining with trypan blue.

4.7. p38 MAPK Overexpression by Plasmid Transfection

In order to increase expression of p38 MAPK in NES2Y cells, transfection with plasmids containing p38 MAPK was performed. The plasmid was originally produced by Roger Davis (Howard Hughes Medical Institute, Chevy Chase, MD, USA). Subsequently, it was subcloned into pcDNA 3.1 by Jarmila Králová (Institute of Molecular Genetics of the ASCR, Prague, Czech Republic) and kindly donated to us (with permission from Davis).

Cells were seeded into 6-well plates at a density of 1×10^6 cells/2.5 mL per well. After 24 h (allowing cells to attach), the culture medium was replaced with new culture medium containing 2.5 µg of plasmid DNA (empty plasmid or plasmid containing p38 MAPK) and Lipofectamine 3000 (Invitrogen, Paisley, UK) as a transfection reagent according to the manufacturer's instructions. After 48 h, the culture medium was replaced with a serum-free medium containing 2% BSA with or without SA. After 18 h of the treatment, cells were harvested and lysates were prepared for Western blot analysis as described previously [8]. The efficiency of transfection was tested by analyzing the level of p38 MAPK using Western blot.

4.8. Assessment of the Effect of p38 MAPK Overexpression on Cell Growth and Viability

The experiment was set up in the same way as that described in "p38 MAPK overexpression by plasmid transfection" with the following modifications. Cells were seeded at a concentration of 2×10^4 cells/100 µL of relevant medium (see above) into the wells of the 96-well plate. The amount of plasmid DNA used was 100 ng per well. After 48 h of incubation with or without SA, the number of living cells was determined using a hemocytometer counting system after staining with trypan blue.

4.9. Inhibitor and Activator Application

Cells (approximately 5×10^5 cells per sample) were seeded and after a 24-h pre-incubation period (allowing cells to attach) the culture medium was replaced with: (1) a serum-free medium with or without the p38 MAPK inhibitor SB202190 (Abcam, Cambridge, UK) at a desired concentration; (2) a serum-free medium containing 2% BSA with or without the p38 MAPK activator anisomycin (Sigma Aldrich, St. Louis, MO, USA) at required concentration; or (3) a serum-free medium containing 2% BSA and SA. The control medium contained only 2% BSA and the *vehiculum* dimethyl sulfoxide (DMSO). After 1 h of inhibitor pre-treatment, 2% BSA with or without SA was added to achieve the required concentrations. After 12 h of incubation, the cells were harvested and lysates were prepared for Western blot analysis as described previously [8]. The concentration of inhibitor/activator, which was necessary for efficient p38 MAPK inhibition/activation, was determined by testing the effect of several inhibitor/activator concentrations on the level of phosphorylated p38 MAPK and/or MAPKAPK-2 (substrate of p38 MAPK). The duration of treatment with the inhibitor/activator was selected based on the time course of activation/inhibition of p38 MAPK after SA and SB202190/anisomycin application.

4.10. Assessment of the Effect of Inhibitor or Activator on Cell Growth and Viability

Cells were seeded at 2×10^4 cells/100 µL of culture media (see above) into the wells of 96-well plate. The p38 MAPK inhibitor SB202190, the activator anisomycin, and SA were applied in the same

way as described above ("Inhibitor and activator application"). After 48 h of incubation, the number of living cells was determined using a hemocytometer counting system after staining with trypan blue.

4.11. Statistical Analysis

The statistical significance of observed differences was determined using the Student´s *t*-test. $p < 0.05$ was considered statistically significant.

Acknowledgments: We thank Roger Davis (Howard Hughes Medical Institute, Chevy Chase, MD, USA) and Jarmila Králová (Institute of Molecular Genetics of the ASCR, Prague, Czech Republic) for providing plasmid DNA. This work was supported by research projects GAUK 1270213, UNCE 204015, and PRVOUK P31 from Charles University in Prague, Czech Republic, and by grant 14-00630P from the Grant Agency of the Czech Republic.

Author Contributions: Jan Šrámek carried out Western blot experiments and wrote the manuscript; Kamila Balušíková and Vlasta Němcová-Fürstová carried out siRNA techniques; Petr Daniel and Michael Jelínek carried out plasmid transfections; Jan Kovář coordinated experiments and helped to complete the manuscript; Roger F. James as well as all authors read and approved the final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Lupi, R.; Dotta, F.; Marselli, L.; del Guerra, S.; Masini, M.; Santangelo, C.; Patane, G.; Boggi, U.; Piro, S.; Anello, M.; *et al.* Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: Evidence that β-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes* **2002**, *51*, 1437–1442. [CrossRef] [PubMed]
2. Maedler, K.; Oberholzer, J.; Bucher, P.; Spinas, G.A.; Donath, M.Y. Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic β-cell turnover and function. *Diabetes* **2003**, *52*, 726–733. [CrossRef] [PubMed]
3. Azevedo-Martins, A.K.; Monteiro, A.P.; Lima, C.L.; Lenzen, S.; Curi, R. Fatty acid-induced toxicity and neutral lipid accumulation in insulin-producing RINm5F cells. *Toxicol. Vitro* **2006**, *20*, 1106–1113. [CrossRef] [PubMed]
4. Welters, H.J.; Diakogiannaki, E.; Mordue, J.M.; Tadayyon, M.; Smith, S.A.; Morgan, N.G. Differential protective effects of palmitoleic acid and cAMP on caspase activation and cell viability in pancreatic β-cells exposed to palmitate. *Apoptosis* **2006**, *11*, 1231–1238. [CrossRef] [PubMed]
5. Fürstova, V.; Kopska, T.; James, R.F.; Kovar, J. Comparison of the effect of individual saturated and unsaturated fatty acids on cell growth and death induction in the human pancreatic β-cell line NES2Y. *Life Sci.* **2008**, *82*, 684–691. [CrossRef] [PubMed]
6. Welters, H.J.; Tadayyon, M.; Scarpello, J.H.; Smith, S.A.; Morgan, N.G. Mono-unsaturated fatty acids protect against β-cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure. *FEBS Lett.* **2004**, *560*, 103–108. [CrossRef]
7. Diakogiannaki, E.; Welters, H.J.; Morgan, N.G. Differential regulation of the endoplasmic reticulum stress response in pancreatic β-cells exposed to long-chain saturated and monounsaturated fatty acids. *J. Endocrinol.* **2008**, *197*, 553–563. [CrossRef] [PubMed]
8. Nemcova-Fürstova, V.; James, R.F.L.; Kovar, J. Inhibitory effect of unsaturated fatty acids on saturated fatty acids-induced apoptosis in human pancreatic β-cells: Activation of caspases and ER stress induction. *Cell. Physiol. Biochem.* **2011**, *27*, 525–538. [CrossRef] [PubMed]
9. Biden, T.J.; Boslem, E.; Chu, K.Y.; Sue, N. Lipotoxic endoplasmic reticulum stress, β cell failure, and type 2 diabetes mellitus. *Trends Endocrinol. Metab.* **2014**, *25*, 389–398. [CrossRef] [PubMed]
10. Cvjeticanin, T.; Stojanovic, I.; Timotijevic, G.; Stosic-Grujicic, S.; Miljkovic, D. T cells cooperate with palmitic acid in induction of β cell apoptosis. *BMC Immunol.* **2009**, *29*, 1–10. [CrossRef] [PubMed]
11. Li, X.L.; Xu, G.; Chen, T.; Wong, Y.S.; Zhao, H.L.; Fan, R.R.; Gu, X.M.; Tong, P.C.; Chan, J.C. Phycocyanin protects INS-1E pancreatic β cells against human islet amyloid polypeptide-induced apoptosis through attenuating oxidative stress and modulating JNK and p38 mitogen-activated protein kinase pathways. *Int. J. Biochem. Cell Biol.* **2009**, *41*, 1526–1535. [CrossRef] [PubMed]
12. Zarubin, T.; Han, J. Activation and signaling of the p38 MAP kinase pathway. *Cell Res.* **2005**, *15*, 11–18. [CrossRef] [PubMed]

13. Ben-Levy, R.; Hooper, S.; Wilson, R.; Paterson, H.F.; Marshall, C.J. Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2. *Curr. Biol.* **1998**, *8*, 1049–1057. [CrossRef]
14. Sakai, N.; Wada, T.; Furuichi, K.; Iwata, Y.; Yoshimoto, K.; Kitagawa, K.; Kokubo, S.; Kobayashi, M.; Takeda, S.; Kida, H.; *et al*. p38 MAPK phosphoryloation and NF-κB activation in human crescentic glomerulonephris. *Nephrol. Dial. Transplant*. **2002**, *17*, 998–1004. [CrossRef] [PubMed]
15. Zhuang, S.; Demirs, J.T.; Kochevar, I.E. p38 mitogen-activated protein kinase mediates bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. *J. Biol. Chem.* **2000**, *275*, 25939–25948. [CrossRef] [PubMed]
16. Berra, E.; Diaz-Meco, M.T.; Moscat, J. The activation of p38 and apoptosis by the inhibition of ERK is antagonized by the phosphoinositide 3-kinase/Akt pathway. *J. Biol. Chem.* **1998**, *273*, 10792–10797. [CrossRef] [PubMed]
17. Cai, B.; Chang, S.H.; Becker, E.B.; Bonni, A.; Xia, Z. p38 MAP kinase mediates apoptosis through phosphorylation of BimEL at Ser-65. *J. Biol. Chem.* **2006**, *281*, 25215–25222. [CrossRef] [PubMed]
18. Yuan, H.; Zhang, X.; Huang, X.; Lu, Y.; Tang, W.; Man, Y.; Wang, S.; Xi, J.; Li, J. NADPH Oxidase 2-derived reactive oxygen species mediate FFAS-induced dysfunction and apoptosis of β-cells via JNK, p38 MAPK and p53 pathways. *PLoS ONE* **2010**, *5*, e15726. [CrossRef] [PubMed]
19. Zhang, Y.; Xu, M.; Zhang, S.; Yan, L.; Yang, C.; Lu, W.; Li, Y.; Cheng, H. The role of G protein-coupled receptor 40 in lipoapoptosis in mouse β-cell line NIT-1. *Mol. Cell. Endocrinol.* **2007**, *38*, 651–661. [CrossRef] [PubMed]
20. Lee, J.; Hong, F.; Kwon, S.; Kim, S.S.; Kim, D.O.; Kang, H.S.; Lee, S.J.; Ha, J.; Kim, S.S. Activation of p38MAPK induces cell cycle arrest via inhibition of c-Raf/ERK pathway during muscle differentiation. *Biochem. Biophys. Res. Commun.* **2002**, *298*, 765–771. [CrossRef]
21. Chang, F.; Steelman, L.S.; Lee, J.T.; Shelton, J.G.; Navolanic, P.M.; Blalock, W.L.; Franklin, R.A.; McCubrey, J.A. Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: Potential targeting for therapeutic intervention. *Leukemia* **2003**, *17*, 1263–1293. [CrossRef] [PubMed]
22. Lu, Z.; Xu, S. ERK1/2 MAP kinases in cell survival and apoptosis. *Life* **2006**, *58*, 621–631. [CrossRef] [PubMed]
23. Huang, D.; Khoe, M.; Befekadu, M.; Chung, S.; Takata, Y.; Ilic, D.; Bryer-Ash, M. Focal adhesion kinase mediates cell survival via NF-κB and ERK signaling pathways. *Am. J. Physiol. Cell Physiol.* **2007**, *292*, C1339–C1352. [CrossRef] [PubMed]
24. Li, D.W.; Liu, J.P.; Mao, Y.W.; Xiang, H.; Wang, J.; Ma, W.Y.; Dong, Z.; Pike, H.M.; Brown, R.E.; Reed, J.C. Calcium-activated RAF/MEK/ERK signaling pathway mediates p53-dependent apoptosis and is abrogated by alpha B-crystallin through inhibition of RAS activation. *Mol. Biol. Cell* **2005**, *16*, 4437–4453. [CrossRef] [PubMed]
25. Zhuang, S.; Yan, Y.; Daubert, R.A.; Han, J.; Schnellmann, R.G. ERK promotes hydrogen peroxide-induced apoptosis through caspase-3 activation and inhibition of Akt in renal epithelial cells. *Am. J. Physiol. Ren. Physiol.* **2007**, *292*, F440–F447. [CrossRef] [PubMed]
26. Heo, J.I.; Oh, S.J.; Kho, Y.J.; Kim, J.H.; Kang, H.J.; Park, S.H.; Kim, H.S.; Shin, J.Y.; Kim, M.J.; Kim, S.C.; *et al*. ERK mediates anti-apoptotic effect through phosphorylation and cytoplasmic localization of p21Waf1/Cip1/Sdi in response to DNA damage in normal human embryonic fibroblast (HEF) cells. *Mol. Biol. Rep.* **2011**, *38*, 2785–2791. [CrossRef] [PubMed]
27. Simon, M.N.; Azevedo-Martins, A.K.; Amanso, A.M.; Carvalho, C.R.O. ; Curim R. Persistent activation of Akt or ERK prevents the toxicity induced by saturated and polyunsaturated fatty acids in RINm5F β-cells. *Toxicol. Vitro* **2008**, *22*, 1018–1024. [CrossRef] [PubMed]
28. Plaisance, V.; Perret, V.; Favrea, D.; Abderrahmania, A.; Yanga, J.-Y.; Widmanna, C.; Regazzi, R. Role of the transcriptional factor C/EBP in free fatty acid-elicited β cell failure. *Mol. Cell. Endocrinol.* **2009**, *305*, 47–55. [CrossRef] [PubMed]
29. Nemcova-Fürstova, V.; Balusikova, K.; Sramek, J.; James, R.F.; Kovar, J. Caspase-2 and JNK Activated by saturated fatty acids are not involved in apoptosis induction but modulate ER stress in human pancreatic β-cells. *Cell. Physiol. Biochem.* **2013**, *31*, 277–289. [CrossRef] [PubMed]
30. Eitel, K.; Staiger, H.; Rieger, J.; Mischak, H.; Brandhorst, H.; Brendel, M.D.; Bretzel, R.G.; Häring, H.U.; Kellerer, M. Protein kinase C Δ activation and translocation to the nucleus are required for fatty acid-induced apoptosis of insulin-secreting cells. *Diabetes* **2003**, *52*, 991–997. [CrossRef] [PubMed]

31. Eitel, K.; Staiger, H.; Brendel, M.D.; Brandhorst, D.; Bretzel, R.G.; Haring, H.U.; Kellerer, M. Different role of saturated and unsaturated fatty acids in β-cell apoptosis. *Biochem. Biophys. Res. Commun.* **2002**, *299*, 853–856. [CrossRef]
32. Cunha, D.A.; Hekerman, P.; Ladriere, L.; Bazarra-Castro, A.; Ortis, F.; Wakeham, M.C.; Moore, F.; Rasschaert, J.; Cardozo, A.K.; Bellomo, E.; *et al*. Initiation and execution of lipotoxic ER stress in pancreatic β-cells. *J. Cell Sci.* **2008**, *121*, 2308–2318. [CrossRef] [PubMed]
33. Lai, E.; Bikopoulos, G.; Wheeler, M.B.; Rozakis-Adcock, M.; Volchuk, A. Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic β-cells. *Am. J. Physiol. Endocrinol. Metab.* **2008**, *294*, E540–E550. [CrossRef] [PubMed]
34. Ladriere, L.; Igoillo-Esteve, M.; Cunha, D.A.; Brion, J.P.; Bugliani, M.; Marchetti, P.; Eizirik, D.L.; Cnop, M. Enhanced signaling downstream of ribonucleic Acid-activated protein kinase-like endoplasmic reticulum kinase potentiates lipotoxic endoplasmic reticulum stress in human islets. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 1442–1449. [CrossRef] [PubMed]
35. Vacaresse, N.; Lajoie-Mazenc, I.; Auge, N.; Suc, I.; Frisach, M.-F.; Salvayre, R.; Nègre-Salvayre, A. Activation of epithelial growth factor receptor pathway by unsaturated fatty acids. *Circ. Res.* **1995**, *85*, 892–899. [CrossRef]
36. Holzer, R.G.; Park, E.J.; Li, N.; Tran, H.; Chen, M.; Choi, C.; Solinas, G.; Karin, M. Saturated fatty acids induce c-Src clustering within membrane subdomains, leading to JNK activation. *Cell* **2011**, *147*, 173–184. [CrossRef] [PubMed]
37. Guo, J.; Qian, Y.Y.; Xi, X.X.; Hu, X.H.; Zhu, J.X.; Han, X. Blockage of ceramide metabolism exacerbates palmitate inhibition of pro-insulin gene expression in pancreatic β-cells. *Mol. Cell. Biochem.* **2010**, *338*, 283–290. [CrossRef] [PubMed]
38. Watson, M.L.; Macrae, K.; Marley, A.E.; Hundal, H.S. Chronic effects of palmitate overload on nutrient-induced insulin secretion and autocrine signalling in pancreatic MIN6 β cells. *PLoS ONE* **2011**, *6*, e25975. [CrossRef] [PubMed]
39. Natalicchio, A.; Labarbuta, R.; Tortosa, F.; Biondi, G.; Marrano, N.; Peschechera, A.; Carchia, E.; Orlando, M.R.; Leonardini, A.; Cignarelli, A.; *et al*. Exendin-4 protects pancreatic β cells from palmitate-induced apoptosis by interfering with GPR40 and the MKK4/7 stress kinase signalling pathway. *Diabetologia* **2013**, *56*, 2456–2466. [CrossRef] [PubMed]
40. Choi, S.E.; Kim, H.E.; Shin, H.C.; Jang, H.J.; Lee, K.W.; Kim, Y.; Kang, S.S.; Chun, J.; Kang, Y. Involvement of Ca^{2+}-mediated apoptotic signals in palmitate-induced MIN6N8a β cell death. *Mol. Cell. Endocrinol.* **2007**, *272*, 50–62. [CrossRef] [PubMed]
41. Johnson, J.D.; Han, Z.; Otani, K.; Ye, H.; Zhang, Y.; Wu, H.; Horikawa, Y.; Misler, S.; Bell, G.I.; Polonsky, K.S. RyR2 and calpain-10 delineate a novel apoptosis pathway in pancreatic islets. *J. Biol. Chem.* **2004**, *279*, 24794–24802. [CrossRef] [PubMed]
42. Maedler, K.; Spinas, G.A.; Dyntar, D.; Moritz, W.; Kaiser, N.; Donath, M.Y. Distinct effects of saturated and monounsaturated fatty acids on β-cell turnover and function. *Diabetes* **2001**, *50*, 69–76. [CrossRef] [PubMed]
43. Maestre, I.; Jordan, J.; Calvo, S.; Reig, J.A.; Cena, V.; Soria, B.; Prentki, M.; Roche, E. Mitochondrial dysfunction is involved in apoptosis induced by serum withdrawal and fatty acids in the β-cell line INS-1. *Endocrinology* **2003**, *144*, 335–345. [CrossRef] [PubMed]
44. Marchetti, P.; del, G.S.; Marselli, L.; Lupi, R.; Masini, M.; Pollera, M.; Bugliani, M.; Boggi, U.; Vistoli, F.; Mosca, F.; *et al*. Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 5535–5541. [CrossRef] [PubMed]
45. Liadis, N.; Salmena, L.; Kwan, E.; Tajmir, P.; Schroer, S.A.; Radziszewska, A.; Li, X.; Sheu, L.; Eweida, M.; Xu, S.; *et al*. Distinct *in vivo* roles of caspase-8 in β-cells in physiological and diabetes models. *Diabetes* **2007**, *56*, 2302–2311. [CrossRef] [PubMed]
46. Shimabukuro, M.; Zhou, Y.T.; Levi, M.; Unger, R.H. Fatty acid-induced β cell apoptosis: A link between obesity and diabetes. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 2498–2502. [CrossRef] [PubMed]
47. Okuyama, R.; Fujiwara, T.; Ohsumi, J. High glucose potentiates palmitate-induced NO-mediated cytotoxicity through generation of superoxide in clonal β-cell HIT-T15. *FEBS Lett.* **2003**, *545*, 219–223. [CrossRef]
48. Jeffrey, K.D.; Alejandro, E.U.; Luciani, D.S.; Kalynyak, T.B.; Hu, X.; Li, H.; Lin, Y.; Townsend, R.R.; Polonsky, K.S.; Johnson, J.D. Carboxypeptidase E mediates palmitate-induced β-cell ER stress and apoptosis. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 8452–8457. [CrossRef] [PubMed]

49. Kharroubi, I.; Ladriere, L.; Cardozo, A.K.; Dogusan, Z.; Cnop, M.; Eizirik, D.L. Free fatty acids and cytokines induce pancreatic β-cell apoptosis by different mechanisms: Role of nuclear factor-κB and endoplasmic reticulum stress. *Endocrinology* **2004**, *145*, 5087–5096. [CrossRef] [PubMed]
50. Rakatzi, I.; Mueller, H.; Ritzeler, O.; Tennagels, N.; Eckel, J. Adiponectin counteracts cytokine- and fatty acid-induced apoptosis in the pancreatic β-cell line INS-1. *Diabetologia* **2004**, *47*, 249–258. [CrossRef] [PubMed]
51. Busch, A.K.; Gurisik, E.; Cordery, D.V.; Sudlow, M.; Denyer, G.S.; Laybutt, D.R.; Hughes, W.E.; Biden, T.J. Increased fatty acid desaturation and enhanced expression of stearoyl coenzyme A desaturase protects pancreatic β-cells from lipoapoptosis. *Diabetes* **2005**, *54*, 2917–2924. [CrossRef] [PubMed]
52. Cnop, M.; Ladrière, L.; Igoillo-Esteve, M.; Moura, R.F.; Cunha, D.A. Causes and cures for endoplasmic reticulum stress in lipotoxic β-cell dysfunction. *Diabetes Obes. Metab.* **2010**, *12*, S76–S82. [CrossRef] [PubMed]
53. Fonseca, S.G.; Gromada, J.; Urano, F. Endoplasmic reticulum stress and pancreatic β-cell death. *Trends Endocrinol. Metab.* **2011**, *22*, 266–274. [CrossRef] [PubMed]
54. Hirosawa, M.; Nakahara, M.; Otosaka, R.; Imoto, A.; Okazaki, T.; Takahashi, S. The p38 pathway inhibitor SB202190 activates MEK/MAPK to stimulate the growth of leukemia cells. *Leuk. Res.* **2009**, *33*, 693–699. [CrossRef] [PubMed]
55. Macfarlane, W.M.; Cragg, H.; Docherty, H.M.; Read, M.L.; James, R.F.; Aynsley-Green, A.; Docherty, K. Impaired expression of transcription factor IUF1 in a pancreatic β-cell line derived from a patient with persistent hyperinsulinaemic hypoglycaemia of infancy (nesidioblastosis). *FEBS Lett.* **1997**, *413*, 304–308. [CrossRef]
56. Musilkova, J.; Kovar, J. Additive stimulatory effect of extracellular calcium and potassium on non-transferrin ferric iron uptake by HeLa and K562 cells. *Biochim. Biophys. Acta* **2001**, *1514*, 117–126. [CrossRef]
57. Kovar, J.; Franek, F. Growth-stimulating effect of transferrin on a hybridoma cell line: Relation to transferrin iron-transporting function. *Exp. Cell Res.* **1989**, *182*, 358–369. [CrossRef]
58. Cnop, M.; Hannaert, J.C.; Hoorens, A.; Eizirik, D.L.; Pipeleers, D.G. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* **2001**, *50*, 1771–1777. [CrossRef] [PubMed]

7.3. PUBLIKACE 3

Šrámek J, Němcová-Fürstová V, Kovář J:
Kinase signaling in apoptosis induced by saturated fatty acid in pancreatic β-cells.
International Journal of Molecular Sciences, 17: 1400, 2016.

International Journal of
Molecular Sciences

MDPI

Review

Kinase Signaling in Apoptosis Induced by Saturated Fatty Acids in Pancreatic β-Cells

Jan Šrámek *, Vlasta Němcová-Fürstová and Jan Kovář *

Division of Cell and Molecular Biology & Center for Research of Diabetes, Metabolism and Nutrition, Third Faculty of Medicine, Charles University in Prague, Ruská 87, Prague 11000, Czech Republic; vlasta.nemcova@lf3.cuni.cz

* Correspondence: jan.sramek@lf3.cuni.cz (J.Š.); jan.kovar@lf3.cuni.cz (J.K.); Tel.: +420-267-102-658 (J.Š. & J.K.)

Academic Editor: Atsushi Matsuzawa
Received: 21 July 2016; Accepted: 22 August 2016; Published: 25 August 2016

Abstract: Pancreatic β-cell failure and death is considered to be one of the main factors responsible for type 2 diabetes. It is caused by, in addition to hyperglycemia, chronic exposure to increased concentrations of fatty acids, mainly saturated fatty acids. Molecular mechanisms of apoptosis induction by saturated fatty acids in β-cells are not completely clear. It has been proposed that kinase signaling could be involved, particularly, c-Jun N-terminal kinase (JNK), protein kinase C (PKC), p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase (ERK), and Akt kinases and their pathways. In this review, we discuss these kinases and their signaling pathways with respect to their possible role in apoptosis induction by saturated fatty acids in pancreatic β-cells.

Keywords: c-Jun N-terminal kinase (JNK); protein kinase C (PKC); p38 mitogen-activated protein kinase (p38 MAPK); extracellular signal-regulated kinase (ERK); Akt; fatty acids; pancreatic β-cell; apoptosis; diabetes

1. Introduction

Increased concentrations of fatty acids (FAs) in blood are known to be one of the main factors responsible for pancreatic β-cell death in type 2 diabetes [1–5]. The detrimental potential of FAs has been described for human as well as animal β-cells in vivo and in vitro [1,2,6–12]. It seems that the toxicity of FAs particularly depends on the degree of their saturation. It was suggested that saturated FAs (e.g., stearic and palmitic acid) induce apoptosis in pancreatic β-cells, whereas the effect of unsaturated FAs (e.g., oleic and palmitoleic acid) on β-cell viability is not entirely clear. It seems that at low concentrations they are well tolerated and are even capable of inhibiting the pro-apoptotic effect of saturated FAs [2,4–6,9,13–16]. Nevertheless, at higher concentrations they might also be pro-apoptotic [17–19]. The precise molecular mechanisms of apoptosis induction by saturated FAs in β-cells remain unclear [20]. However, it has been proposed that kinase signaling pathways could be involved [10,21–23].

Saturated FAs were shown to induce endoplasmic reticulum (ER) stress in cells including pancreatic β-cells. ER stress was demonstrated to result in activation of signaling pathways starting mainly with three membrane proteins, i.e., inositol-requiring protein 1α (IRE1α), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6). Activation of IRE1α leads to c-Jun N-terminal kinase (JNK) activation by phosphorylation, which further phosphorylates c-Jun. The mentioned signaling pathways primarily participate in the restoration of ER homeostasis. However, if this response fails, apoptosis is induced by mechanisms that are not still completely understood (reviewed in [20,24]).

Kinase signaling pathways are regulated in response to various extracellular physical (e.g., UV radiation, and temperature) and chemical (many agens) stimuli and also in response to various

cytokines. They can be involved, depending on cell type, in the regulation of many cellular processes such as proliferation, differentiation, inflammatory response, autophagy, senescence, and also in apoptosis (reviewed in [25]).

In this review, we will discuss kinase signaling pathways with a possible role in apoptosis induction by saturated FAs in pancreatic β-cells. Concerning this, JNK, protein kinase C (PKC), p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2), and Akt (also known as protein kinase B (PKB) kinase) signaling have been the most extensively studied [26–28]. Thus, we will discuss available data on above-mentioned pathways, from both in vitro as well as in vivo experiments using β-cells of animal (mainly rat and murine) and human origin.

2. c-Jun N-Terminal Kinase (JNK)

2.1. JNK and Its Role in Cell Signaling

JNK is a serine-threonine kinase. It was described in the early 1990s [29,30] when three isoforms were identified, i.e., JNK1, JNK2, and JNK3 (also referred to as stress-activated protein kinase (SAPK)-γ, SAPK-α and SAPK-β, respectively) [31–33]. JNK is activated by mitogen-activated protein kinase kinase (MKK) 4 and MKK7 via dual phosphorylation on the tripeptide motif Thr-Pro-Tyr. This tripeptide is located within the activation T-loop in protein kinase subdomain VIII [34]. MKK4 and MKK7 are activated by several MAP kinase kinase kinases (MAP3Ks) as e.g., transforming growth factor-β-activated kinase 1 (TAK1), apoptosis signal-regulating kinase 1 (ASK1), tumor progression locus 2 (TPL2), and mixed-lineage kinases (MLKs) and by some members of the MEKK family. Besides this mechanism, JNK kinase can also be activated by IRE1α protein [35], which represents one of the main signaling pathways of ER stress. It has been showed that ER stress can mediate apoptosis induction by different stimuli including FAs [20,24].

JNK can affect the function of many proteins (reviewed in [36]) including transcription factors (e.g., signal transducers and activators of transcription (STAT), p53, and proteins of forkhead box (Foxo) or ATF family), mitochondrial proteins (e.g., Sab or proteins of B-cell lymphoma 2 (Bcl-2) family), other protein kinases (Akt, p90 ribosomal S6 kinase (p90RSK)), and proteins associated with cell movement (kinesin, doublecortin) or protein degradation (E3 ligase). JNK kinase is mostly involved in mediating the apoptotic response of cells to pro-inflammatory cytokines, and genotoxic or environmental stresses. However, activation of this kinase is also associated with the regulation of cell proliferation, differentiation, survival, and autophagy [34].

2.2. JNK in Apoptosis Induced by Saturated Fatty Acids in β-Cells

In Sand rat islets as well as in INS-1 and INS-1E rat β-cells, several authors found that JNK is activated, and mediate apoptosis induction by palmitate or palmitate in combination with high glucose concentrations [22,27,37–41]. We showed that JNK is activated in INS-1E cells in response to pro-apoptotic stearate application (unpublished data). Contrary to previous data, it was also shown that the JNK1 isoform protects against FA/high glucose-induced apoptosis in these cells [42].

Regarding murine pancreatic β-cells, Abaraviciene et al. (2008) [43] as well as Natalicchio et al. (2013) [37] demonstrated in primary islets that JNK activation is involved in palmitate-induced pro-apoptotic signaling. Similar data were obtained in MIN6 and MIN6N8 cells [17,44]. In NIT-1 cells, Yuan et al. (2010) [19] also found that JNK mediates apoptosis induction by palmitate. However, this was not confirmed by Zhang et al. (2007) [26] who documented no effects of apoptosis-inducing palmitate treatment on JNK activation in these cells. Nevertheless, exposure time was only 10 min in their experiment.

In human primary islets, it was demonstrated that JNK activation mediates the pro-apoptotic effects of palmitate [37]. The pro-apoptotic role of this kinase in human islets has also been demonstrated by Aikin et al. (2004) [45]. Authors have also shown that JNK can be inhibited by phosphoinositide 3-kinase (PI3K)/Akt signaling. However, in NES2Y human β-cells, it seems that

stearic acid-induced JNK activation, which is associated with the stearic acid-induced ER stress marker immunoglobulin heavy chain-binding protein (BiP), is probably not a key step in apoptosis induction by stearate [46].

It is generally accepted that JNK kinase is activated by saturated FAs-induced ER stress in pancreatic β-cells [20,38].

To conclude, it has been documented that saturated FAs activate JNK kinase in pancreatic β-cells mainly via ER stress. However, other mechanisms cannot be excluded. It seems that JNK activation mediates apoptosis induction by saturated FAs. Nevertheless, the amount of available data is insufficient to say that this is conclusive. In addition to the process of apoptosis induction, JNK is probably also involved in fatty acid-induced autophagy [47] in pancreatic β-cells. The pro-apoptotic role of JNK has also been documented in osteoblasts and hepatocytes treated with saturated FAs [48–50]. JNK seems to be one of the most important kinases in the process of apoptosis induction by FAs in pancreatic β-cells, mainly via ER stress induction (Figure 1).

3. Protein Kinase C (PKC)

3.1. PKC and Its Role in Cell Signaling

The PKC family of serine-threonine kinases comprises at least ten isoforms that are divided into three groups based on sequence homology and mechanism of activation [51]: (1) conventional PKC (PKCα, β1, β2 and γ); (2) novel PKC (PKCδ, ε, η and θ); and (3) atypical PKC (PKCζ and PKCι/λ). The first two groups are activated by diacylglycerol (DAG) in response to receptor signaling coupled with the activation of phospholipase C. Novel PKC isoforms can be also activated by DAG but are Ca^{2+} insensitive. Atypical PKCs are not activated by DAG and Ca^{2+} but instead in response to receptors that stimulates PI3K leading to activation of phosphoinositide-dependent protein kinase 1 (PDK1).

PKC isoforms have been associated with numerous physiological functions, including secretion and exocytosis, modulation of ion conductance, cell proliferation, and apoptosis or survival [52–54]. The PKCδ isoform was the first identified member of the novel PKC group. This isoform was, of the respective PKC isoforms, the most often found to be connected with the process of apoptosis induction via regulation of its down-stream targets, e.g., JNK-STAT1 signaling, Akt, ERK1/2, and p38 MAPK kinases [55–58].

3.2. PKC in Apoptosis Induced by Saturated Fatty Acids in β-Cells

In the rat INS-1 cell line, it has been shown that PKCδ activation can mediate apoptosis induction by palmitate [23]. Wrede et al. (2003) [28] demonstrated, using the same cell line, the activation of PKCδ by long-chain acyl-CoA. The authors also showed that PKCδ can inhibit Akt kinase. In addition, our unpublished data concerning activation of PKCδ in response to pro-apoptotic stearic acid also suggests that PKCδ is involved in saturated FAs-induced apoptosis in INS-1E cells. In RIN1046-38 rat β-cells, PKCδ activation and translocation from cytosol to the nucleus was found to be necessary for apoptosis induction by saturated FAs (palmitate, stearate) in contrast to unsaturated FAs (palmitoleate, oleate, linoleate) [59]. Similarly, Simon et al. (2008) [60] reported, in rat RINm5F β-cells, that PKCδ kinase was activated in response to low, but still apoptosis-inducing, concentrations of palmitic acid and non-apoptosis-inducing concentrations of stearic acid. The authors suggested that PKCδ mediates apoptosis induction by these FAs. Alcáazar et al. (1997) [61] observed, in Langerhans islets of Wistar albino rats, a translocation of PKC activity (respective isoforms were not tested) from cytosol to cellular membranes after palmitate and high glucose co-application. However, they did not test whether PKC was a mediator of apoptosis induction under these experimental conditions. On the other hand, it seems that in the BRIN-BD11 rat cell line, PKCδ is not required for palmitate-induced apoptosis [9].

Hennige et al. (2010) [12] showed in murine pancreatic β-cells in vivo that overexpression of dominant-negative PKCδ protects against high fat diet-induced β-cell failure through a mechanism that involves inhibition of FA-mediated apoptosis. Furthermore, Qi et al. (2010) [18] documented

activation of PKC α and β1 isoforms after exposure to apoptosis-inducing concentrations of palmitate in MIN6 β-cells, which suggests a pro-apoptotic role for the tested isoforms.

With regard to human pancreatic β-cells, there is only one piece of data available. We found no effect on PKCδ activation resulting from treatment with pro-apoptotic concentrations of stearate in NES2Y cells (unpublished data).

Generally, it seems that PKCδ is activated by saturated FAs and it can mediate apoptosis induction by saturated FAs in pancreatic β-cells. However, this role for PKCδ has not been demonstrated in human β-cells, till now (our unpublished data). Nuclear localization might be necessary for pro-apoptotic function of PKCδ. The involvement of PKCδ activation in apoptosis induction in pancreatic β-cells by stimuli other than FAs, such as interleukin 1β (IL-1β) and streptozotocin, has been described [62]. The pro-apoptotic role of PKCδ has been found in a variety of cell types [63–65] and is now generally accepted [54]. On the other hand, the α, β, ε, λ, and ζ isoforms of PKC seem to be pro-survival [54]. PKCδ can also inhibit Akt kinase in β-cells. PKCδ seems to be important in the process of apoptosis induction by FAs in pancreatic β-cells, similar to JNK and p38 MAPK (Figure 1).

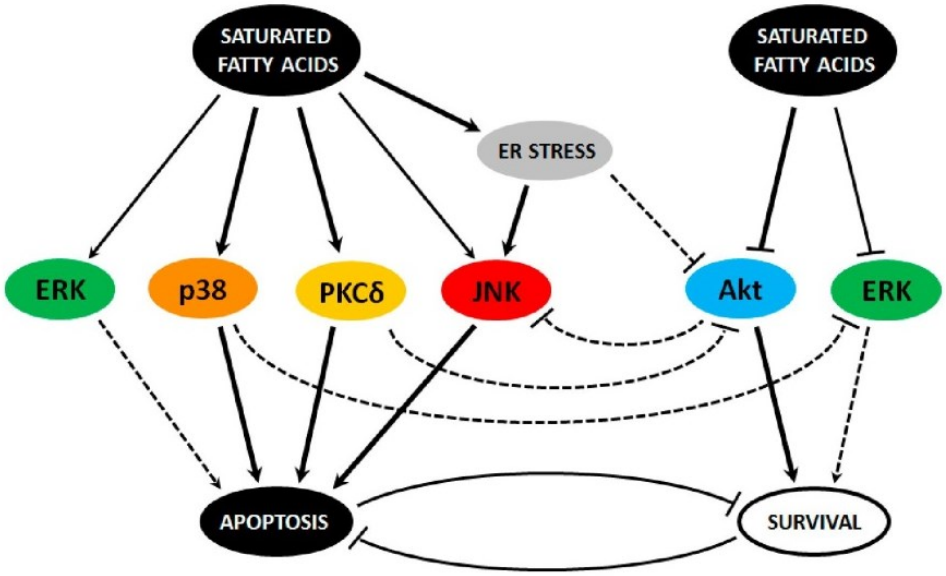


Figure 1. The involvement of c-Jun N-terminal kinase (JNK), protein kinase C (PKC), p38 mitogen-activated protein kinase (p38 MAPK), extracellular signal-regulated kinase (ERK), and Akt kinases and their pathways in apoptosis induction by saturated fatty acids (FAs) in pancreatic β-cells. Solid lines represent relationships with a reasonable probability where bold solid line means more important relationship. Dashed lines represent possible, but less certain, relationships.

4. p38 Mitogen-Activated Protein Kinase (p38 MAPK)

4.1. p38 MAPK and Its Role in Cell Signaling

To date, four splice variants of the p38 MAPK serine-threonine kinase have been identified: p38α, p38β [66], p38γ [67,68], and p38δ [69,70]. p38 MAPK is mainly activated, as is JNK, by MKK3/6 kinases or, in some specific cell types, by MKK4 kinase. Several MKK kinases (MAP3Ks) have been identified as upstream activators. These include TAK1 [71], ASK1 [72], MLKs [73], and some members of the MEKK family [74,75]. GTP-binding proteins from the Rho subfamily, such as Ras-related C3 botulinum toxin substrate 1 (Rac1), cell division control protein 42 (Cdc42), or Rho [73,76], can contribute to activation of p38 MAPK upstream from MAP3Ks. GTP-binding proteins are activated in response to various extracellular physical (UV light, heat) and chemical (anisomycin) stimuli, and cytokines (tumor necrosis factor α (TNF-α), colony stimulating factor 1 (CSF-1)) (reviewed in [77]).

p38 MAPK can, depending on cell type, regulate activation of various proteins such as transcription factors (CCAAT-enhancer-binding protein C/EBP (CHOP), and activating transcription factor 2 (ATF2)), other protein kinases (MAP kinase-activated protein kinase (MAPKAPK)-2 and -3, and mitogen- and stress-activated protein kinase 1/2 (MSK1/2)), and translation machinery components (eukaryotic translation initiation factor 4E (eIF-4E)) (reviewed in [78]). This signaling pathway can regulate, depending on cell type, different processes including cell cycle, differentiation, inflammation, senescence, autophagy, and apoptosis [77,79].

4.2. p38 MAPK in Apoptosis Induced by Saturated Fatty Acids in β-Cells

It has been showed in rat pancreatic islets and insulinoma cells (RIN) as well as in rat INS-1E cells that p38 MAPK activation mediates apoptosis induction by palmitate [21,37]. Additionally, we have also shown activation of the p38 MAPK pathway (MKK3/6→p38 MAPK→MAPKAPK-2) in INS-1E cells in response to treatment with pro-apoptotic concentrations of stearic acid (unpublished data).

p38 MAPK activation is involved in palmitate-induced pro-apoptotic signaling in murine primary islets [37,43]. Yuan et al. (2010) [19] obtained similar data in mouse NIT-1 β-cells while Zhang et al. (2007) [26] was unable to confirm these results. They found no effect of apoptosis-inducing concentrations of palmitate treatment on p38 MAPK activation in these cells. However, Zhang et al. (2007) analyzed the FA effect after only 10-min of exposure, compared to 48 h of exposure used by Yuan et al. (2010) [19].

It has been shown in human primary islets that p38 MAPK activation mediates the pro-apoptotic effects of palmitate [37]. Activation of the p38 MAPK pathway (MKK3/6→p38 MAPK→MAPKAPK-2), in response to treatment with pro-apoptotic concentrations of stearate, has been documented in NES2Y human β-cells. It has been shown that this activation could be involved in apoptosis induction by stearic acid; however, this involvement does not seem to play a key role. It has also been shown that activated p38 MAPK probably inhibits the ERK pathway [80].

Concerning a possible connection between p38 MAPK and ER stress signaling in pancreatic β-cells, there is relatively little data available suggesting that p38 MAPK activation functions upstream of ER stress [81].

In summary, it seems that p38 MAPK is activated by saturated FAs and is somehow involved in pro-apoptotic signaling induced by saturated FAs in animal as well as human pancreatic β-cells. Such function of p38 MAPK activation was also demonstrated in rodent and human islets treated by oxidized lipids [82] and pro-apoptotic cytokines [83,84]. The pro-apoptotic role of p38 MAPK signaling has been suggested in other cell types treated with FAs, e.g., coronary artery endothelial cells [85], vascular smooth muscle cells [86], and hepatocytes [87]. p38 MAPK, when activated by saturated FAs in pancreatic β-cells, seems to inhibit ERK1/2 kinase. p38 MAPK seems to be one of the most important kinases in the process of apoptosis induction by FAs in pancreatic β-cells, similar to JNK and PKCδ (Figure 1).

5. Extracellular Signal-Regulated Kinase 1/2 (ERK1/2)

5.1. ERK1/2 and Its Role in Cell Signaling

ERK1/2 (proteins ERK1 and ERK2 with 85% sequence homology, also known as p42/p44 MAPK) are serine-threonine kinases and are well-known members of the MAPK kinase family. It is a part of the c-Raf→MEK1/2→ERK1/2 signaling pathway, i.e., the ERK pathway. This pathway, depending on cell type, is activated through a variety of different stimuli, including growth factors and cytokines via the relevant receptor tyrosine kinases (RTKs) [88], and ligands of heterotrimeric G protein-coupled receptors [89]. Subsequently, Ras GTPase is activated and recruits c-Raf kinase into a complex where it becomes activated [90]. Then, c-Raf phosphorylates the mitogen-activated protein kinase/ERK kinase (MEK1/2), which in turn activates ERK1/2. c-Raf kinase can be activated, not only by Ras protein, but also by other proteins such as PKC [91], Src [92], and p21-activated protein kinase (PAK) [93].

Depending on the cell type, ERK1/2 kinase regulates the function of numerous substrates across all cellular compartments including several kinases (ribosomal S6 kinase (RSKs), MSKs, and MAP kinase signal-integrating kinases (MNKs)), cytoskeletal proteins (paladin and paxillin), transcription factors (c-Fos, and c-Myc), and membrane proteins (spleen tyrosine kinase (Syk), and calnexin) (reviewed in [94]). The ERK signaling pathway is generally considered to be a key regulator of cell proliferation, however, it may also be involved in the process of differentiation [95], autophagy, senescence, and apoptosis (reviewed in [96]).

5.2. ERK1/2 in Apoptosis Induced by Saturated Fatty Acids in β-Cells

Quan et al. (2014) [27] reported that, in the rat INS-1 β-cell line, treatment with apoptosis-inducing concentrations of palmitate led to inhibition of the MEK1/2-ERK1/2 signaling. The authors demonstrated that the signaling had a pro-survival function in INS-1 cells. Guo et al. (2010) [97], using the same cells, also showed ERK1/2 inhibition, even after very brief treatment (10 min) with non-apoptosis-inducing concentrations of palmitate. We have observed activation of the ERK pathway, in INS-1E cells, following exposure to apoptosis-inducing concentrations of stearate (unpublished data). Simon et al. (2008) [60], in RINm5F cells, found activation of this kinase in response to short-term, but not to long-term, treatment with low, but apoptosis-inducing concentrations of palmitic acid and non-toxic concentrations of stearic acids. The authors suggested that persistent ERK1/2 activation via saturated FAs may have pro-survival functions to prevent the toxic effects of late PKCδ activation. Since survival pathways are activated, this may explain why short-period exposure to FA is not enough to induce apoptosis in pancreatic β-cells.

Plaisance et al. (2009) [98], in murine MIN6 β-cells, showed that ERK1/2 activation is involved in palmitate-induced apoptosis. Activation of ERK1/2 in response to non-apoptosis-inducing concentrations of palmitate was also documented by Fontés et al. (2009) [99] in the same cell line as well as in rat islets. Watson et al. (2011) [100], on the other hand, showed inhibition of ERK1/2 after exposure to pro-apoptotic concentrations of palmitate in MIN6 cells. Abaraviciene et al. (2008) [43] demonstrated ERK1/2 activation in murine primary islets in response to short-term (30 min) treatment with palmitate at apoptosis-inducing concentrations. Longer incubation (24 h) with palmitate had no effect on ERK1/2 activation.

Concerning human pancreatic β-cells, it has been shown in the NES2Y cell line that apoptosis-inducing stearic acid inhibits the ERK pathway, probably via p38 MAPK activation. However, this ERK pathway inhibition does not seem to be essential for stearate-induced apoptosis [80].

A possible connection between ERK1/2 and ER stress signaling in β-cells was also tested, however, with negative results ([81], our unpublished data).

Taken together, the data show that saturated FAs in pancreatic β-cells in some cases stimulate and in some cases inhibit ERK1/2, i.e., the ERK pathway. Reasons for these rather confusing findings are unclear. In the case of ERK1/2 stimulation, it can result in both pro-survival as well as pro-apoptotic effects. Again, these findings are rather confusing. In the case of ERK1/2 inhibition, it seems that the pro-survival effects of ERK1/2 are inhibited. However, we should note that we are dealing with relatively little data. It has been documented that ERK1/2 activation can mediate apoptosis induction by IL-1β or 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) dioxin in pancreatic β-cells and/or rat islets [101–104]. On the other hand, there are other studies that document the pro-survival functions of the ERK pathway in pancreatic β-cells as well as in mice and human islets [105,106]. In other cell types, ERK1/2 (the ERK pathway) can also have both pro-survival [107] as well as pro-apoptotic [108,109] functions. We should mention that in β-cells treated with saturated FAs, ERK1/2 seems to be inhibited by p38 MAPK. In comparison with the other discussed kinases, ERK1/2 does not play a decisive role in apoptosis induction by FAs in pancreatic β-cells (Figure 1).

6. Akt (PKB, Protein Kinase B)

6.1. Akt and Its Role in Cell Signaling

There are three known isoforms of Akt kinase, i.e., Aktα (Akt1), Aktβ (Akt2), and Aktγ (Akt3). The pathway leading to the activation of this serine-threonine kinase starts with the interaction of the respective ligands with various RTKs, G-protein coupled receptors, and integrins that activate PI3K. This kinase consequently activates PDK1 through phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) (reviewed in [110]).

PDK1 further activates Akt kinase, which in turn regulates activation of many substrates involved in cell growth and proliferation, e.g., mammalian target of rapamycin (mTOR) and cyclins (reviewed in [111]), angiogenesis via endothelial nitric oxide synthase (eNOS), (reviewed in [112]), and apoptosis (e.g., FoxO1, Bcl-2-associated death promoter (BAD) or nuclear factor κB (NF-κB) [113–115]).

6.2. Akt in Apoptosis Induced by Saturated Fatty Acids in β-Cells

Higa et al. (2006) [10], in the INS-1 cell line, found that Akt activation could mediate apoptosis induction by palmitate. Qin et al. (2014) [23] documented activation of Akt in response to apoptosis-inducing palmitate treatment in the same cell line. We also observed Akt activation due to the application of apoptosis-inducing stearic acid, in INS-1E cells (unpublished data). Fontés et al. (2009) [99] demonstrated in rat islets that cytotoxic palmitate treatment increases Akt phosphorylation. On the contrary, several authors [22,27,40], using similar concentrations and exposure times as in discussed experiments, found that treatment with apoptosis-inducing concentrations of palmitate led to Akt inhibition. They also showed pro-survival function of this kinase in INS-1 cell line. In RINm5F rat β-cells, Simon et al. (2008) [60] showed inhibition of Akt in response to low (0.1 mM) but apoptosis-inducing concentrations of palmitic acid and non-apoptosis-inducing concentrations of stearic acid. The authors suggested that Akt probably has pro-survival function here. A similar role for Akt kinase, in these cells, was also suggested by Nicoletti-Carvalho et al. (2010) [116] who documented that palmitate at apoptosis-inducing concentrations led to inhibition of interleukin-6-activated Akt. However, it is interesting that the application of this acid alone inhibited Akt only very slightly.

Li et al. (2012) [117] demonstrated in murine MIN6 pancreatic β-cells that treatment with cytotoxic concentrations of palmitate led to inhibition of Akt phosphorylation followed by FoxO1 nuclear re-distribution. They demonstrated PI3K/Akt pro-survival and FoxO1 pro-apoptotic functions in these cells. Similar data have also been obtained by other authors [118,119]. Watson et al. (2011) [100] showed that Akt kinase was inhibited after exposure to palmitate at apoptosis-inducing concentrations. Interestingly, Fontés et al. (2009) [99] found in MIN6 cells, that treatment with pro-apoptotic concentrations of palmitate increases Akt phosphorylation 12–18 h after application but not after 24 h. The authors hypothesized that under non-pro-apoptotic conditions, FAs enhances Akt phosphorylation. When either the concentration or the length of exposure is increased, FAs-induced cell death is associated with a secondary decrease in Akt phosphorylation. This idea can be supported by data from Martinez et al. (2008) [17] who demonstrated, in MIN6 cells, that early enhancement of Akt phosphorylation by apoptosis-inducing concentrations of palmitate at 4 h was followed by a decrease at 24 h and was associated with cell death. In the NIT-1 mouse cell line, activation of Akt kinase in response to short-term exposure to pro-apoptotic concentrations of palmitate has been demonstrated [26].

With regard to human pancreatic β-cells, the only available data are our unpublished data. We documented Akt kinase inhibition in NES2Y cells in response to apoptosis-inducing concentrations of stearate. However, we did not test whether this inhibition was related to stearate-induced apoptosis.

It was showed that under pathological conditions of chronic activation, ER stress inhibits Akt kinase in pancreatic β-cells [40,120,121]. Akt can also be inhibited by PKCδ [28] and, on the other hand, it can inhibit JNK [45] in β-cells.

Based on the above data, it seems (in spite of some contradictory results, especially in INS-1 cells) that the Akt kinase/pathway is inhibited by saturated FAs and has a pro-survival function in pancreatic β-cells. The pro-survival role of this kinase in β-cells is supported by the findings of Tuttle et al. (2001) [122] that Akt kinase increases β-cell size and mass in C57BL/6J mice and of Aikin et al. (2004) [45] that PI3K/Akt signaling suppresses the pro-apoptotic JNK pathway in human islets. The pro-survival function of Akt kinase/pathway has been documented in other cell types, too (e.g., [123,124]). Additionally, Akt can be inhibited by PKCδ in β-cells.

7. Conclusions

According to available data, it seems that JNK, PKCδ, and p38 MAPK kinases and their pathways are activated, JNK via ER stress, by saturated FAs. These kinases probably represent main kinase signaling mediating apoptosis inducted by saturated FAs in pancreatic β-cells. Another important kinase signaling pathway seems to be Akt, which is inhibited by saturated FAs, possibly through ER stress, and has what appear to be pro-survival functions in pancreatic β-cells. The effect of saturated FAs on ERK1/2 and the role of its pathway, in β-cells, after saturated FAs exposure are not completely clear since available data tend to be rather contradictory. The ERK pathway may be activated as well as inhibited by saturated FAs. Similarly, the role of this pathway can be pro-apoptotic as well as pro-survival in pancreatic β-cells. There might be crosstalks between some of the discussed pathways (Figure 1).

Acknowledgments: This work was supported by research projects GAUK 1270213, UNCE 204015, and PRVOUK P31 from Charles University in Prague, Czech Republic, and by grant 14-00630P from the Grant Agency of the Czech Republic.

Author Contributions: Jan Šrámek wrote the manuscript. Vlasta Němcová-Fürstová and Jan Kovář improved the text of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ASK1	apoptosis signal-regulating kinase 1
ATF2	activating transcription factor 2
ATF6	activating transcription factor 6
BAD	Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma 2
BiP	immunoglobulin heavy chain-binding protein
Cdc42	cell division control protein 42
CHOP	CCAAT-enhancer-binding protein C/EBP
CSF-1	colony stimulating factor 1
DAG	diacylglycerol
eIF-4E	eukaryotic translation initiation factor 4E
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinase 1/2
FA	fatty acid
Foxo	forkhead box
IL-1β	interleukin 1β
IRE1α	inositol-requiring protein 1α
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MAPKAPK	MAP kinase-activated protein kinase
MEK1/2	mitogen-activated protein kinase/ERK kinase
MKK	mitogen-activated protein kinase kinase
MLKs	mixed-lineage kinases
MNKs	MAP kinase signal-integrating kinases
MNKs	MAP kinase signal-integrating kinases
MSK1/2	mitogen- and stress-activated protein kinase 1/2
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor κB

PAK	p21-activated protein kinase
PERK	protein kinase RNA (PKR)-like ER kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PI3K	phosphoinositide 3-kinase
PDK1	phosphoinositide-dependent protein kinase 1
PKB	protein kinase B (also known as Akt kinase)
PKC	protein kinase C
p90RSK	p90 ribosomal S6 kinase
Rac1	Ras-related C3 botulinum toxin substrate 1
Raf	rapidly accelerated fibrosarcoma
Ras	rat sarcoma
RTKs	receptor tyrosine kinases
SAPK	stress-activated protein kinase
STAT	signal transducers and activators of transcription
Syk	spleen tyrosine kinase
TAK1	transforming growth factor-β-activated kinase 1
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TNF-α	tumor necrosis factor α
TPL2	tumor progression locus 2

References

1. Lupi, R.; Dotta, F.; Marselli, L.; del Guerra, S.; Masini, M.; Santangelo, C.; Patane, G.; Boggi, U.; Piro, S.; Anello, M.; et al. Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: Evidence that β-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes* **2002**, *51*, 1437–1442. [CrossRef] [PubMed]
2. Maedler, K.; Oberholzer, J.; Bucher, P.; Spinas, G.A.; Donath, M.Y. Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic β-cell turnover and function. *Diabetes* **2003**, *52*, 726–733. [CrossRef] [PubMed]
3. Azevedo-Martins, A.K.; Monteiro, A.P.; Lima, C.L.; Lenzen, S.; Curi, R. Fatty acid-induced toxicity and neutral lipid accumulation in insulin-producing RINm5F cells. *Toxicol. In Vitro* **2006**, *20*, 1106–1113. [CrossRef] [PubMed]
4. Welters, H.J.; Diakogiannaki, E.; Mordue, J.M.; Tadayyon, M.; Smith, S.A.; Morgan, N.G. Differential protective effects of palmitoleic acid and cAMP on caspase activation and cell viability in pancreatic β-cells exposed to palmitate. *Apoptosis* **2006**, *11*, 1231–1238. [CrossRef] [PubMed]
5. Fürstova, V.; Kopska, T.; James, R.F.; Kovar, J. Comparison of the effect of individual saturated and unsaturated fatty acids on cell growth and death induction in the human pancreatic β-cell line NES2Y. *Life Sci.* **2008**, *82*, 684–691. [CrossRef]
6. Maedler, K.; Spinas, G.A.; Dyntar, D.; Moritz, W.; Kaiser, N.; Donath, M.Y. Distinct effects of saturated and monounsaturated fatty acids on β-cell turnover and function. *Diabetes* **2001**, *50*, 69–76. [CrossRef] [PubMed]
7. El-Assaad, W.; Buteau, J.; Peyot, M.L.; Nolan, C.; Roduit, R.; Hardy, S.; Joly, E.; Dbaibo, G.; Rosenberg, L.; Prentki, M. Saturated fatty acids synergize with elevated glucose to cause pancreatic β-cell death. *Endocrinology* **2003**, *144*, 4154–4163.
8. Kharroubi, I.; Ladriere, L.; Cardozo, A.K.; Dogusan, Z.; Cnop, M.; Eizirik, D.L. Free fatty acids and cytokines induce pancreatic β-cell apoptosis by different mechanisms: role of nuclear factor-κB and endoplasmic reticulum stress. *Endocrinology* **2004**, *145*, 5087–5096. [CrossRef] [PubMed]
9. Welters, H.J.; Tadayyon, M.; Scarpello, J.H.; Smith, S.A.; Morgan, N.G. Mono-unsaturated fatty acids protect against β-cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure. *FEBS Lett.* **2004**, *560*, 103–108. [CrossRef]
10. Higa, M.; Shimabukuro, M.; Shimajiri, Y.; Takasu, N.; Shinjyo, T.; Inaba, T. Protein kinase B/Akt signaling is required for palmitate-induced β-cell lipotoxicity. *Diabetes Obes. Metab.* **2006**, *8*, 228–233. [CrossRef]

11. Karaskov, E.; Scott, C.; Zhang, L.; Teodoro, T.; Ravazzola, M.; Volchuk, A. Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic β-cell apoptosis. *Endocrinology* **2006**, *147*, 3398–3407. [CrossRef] [PubMed]
12. Hennige, A.M.; Ranta, F.; Heinzelmann, I.; Dufer, M.; Michael, D.; Braumuller, H.; Lutz, S.Z.; Lammers, R.; Drews, G.; Bosch, F.; et al. Overexpression of kinase-negative protein kinase Cδ in pancreatic β-cells protects mice from diet-induced glucose intolerance and β-cell dysfunction. *Diabetes* **2010**, *59*, 119–127. [CrossRef] [PubMed]
13. Eitel, K.; Staiger, H.; Brendel, M.D.; Brandhorst, D.; Bretzel, R.G.; Haring, H.U.; Kellerer, M. Different role of saturated and unsaturated fatty acids in β-cell apoptosis. *Biochem. Biophys. Res. Commun.* **2002**, *299*, 853–856. [CrossRef]
14. Sone, H.; Kagawa, Y. Pancreatic β cell senescence contributes to the pathogenesis of type 2 diabetes in high-fat diet-induced diabetic mice. *Diabetologia* **2005**, *48*, 58–67. [CrossRef] [PubMed]
15. Diakogiannaki, E.; Welters, H.J.; Morgan, N.G. Differential regulation of the endoplasmic reticulum stress response in pancreatic β-cells exposed to long-chain saturated and monounsaturated fatty acids. *J. Endocrinol.* **2008**, *197*, 553–563. [CrossRef] [PubMed]
16. Nemcova-Fürstova, V.; James, R.F.L.; Kovar, J. Inhibitory effect of unsaturated fatty acids on saturated fatty acids-induced apoptosis in human pancreatic β-cells: Activation of caspases and ER stress induction. *Cell. Physiol. Biochem.* **2011**, *27*, 525–538. [CrossRef] [PubMed]
17. Martinez, S.C.; Tanabe, K.; Cras-Méneur, C.; Abumrad, N.A.; Bernal-Mizrachi, E.; Permutt, M.A. Inhibition of FoxO1 protects pancreatic islet β-cells against fatty acid and endoplasmic reticulum stress-induced apoptosis. *Diabetes* **2008**, *57*, 846–859. [CrossRef] [PubMed]
18. Qi, D.; Cai, K.; Wang, O.; Li, Z.; Chen, J.; Deng, B.; Qian, L.; Le, Y. Fatty acids induce amylin expression and secretion by pancreatic β-cells. *Am. J. Physiol. Endocrinol. Metab.* **2010**, *298*, E99–E107. [CrossRef] [PubMed]
19. Yuan, H.; Zhang, X.; Huang, X.; Lu, Y.; Tang, W.; Man, Y.; Wang, S.; Xi, J.; Li, J. NADPH Oxidase 2-Derived Reactive Oxygen Species Mediate FFAs-Induced Dysfunction and Apoptosis of β-Cells via JNK, p38 MAPK and p53 Pathways. *PLoS ONE* **2010**, *5*, e15726. [CrossRef] [PubMed]
20. Biden, T.J.; Boslem, E.; Chu, K.Y.; Sue, N. Lipotoxic endoplasmic reticulum stress, β cell failure, and type 2 diabetes mellitus. *Trends Endocrinol. Metab.* **2014**, *25*, 389–398. [CrossRef] [PubMed]
21. Cvjeticanin, T.; Stojanovic, I.; Timotijevic, G.; Stosic-Grujicic, S.; Miljkovic, D. T cells cooperate with palmitic acid in induction of β cell apoptosis. *BMC Immunol.* **2009**, *29*, 1–10. [CrossRef] [PubMed]
22. Lee, J.H.; Jung, I.R.; Choi, S.E.; Lee, S.M.; Lee, S.J.; Han, S.J.; Kim, H.J.; Kim, D.J.; Lee, K.W.; Kang, Y. Toxicity generated through inhibition of pyruvate carboxylase and carnitine palmitoyl transferase-1 is similar to high glucose/palmitate-induced glucolipotoxicity in INS-1 β cells. *Mol. Cell. Endocrinol.* **2014**, *383*, 48–59. [CrossRef] [PubMed]
23. Qin, J.; Fang, N.; Lou, J.; Zhang, W.; Xu, S.; Liu, H.; Fang, Q.; Wang, Z.; Liu, J.; Men, X.; et al. TRB3 Is Involved in Free Fatty Acid-Induced INS-1-Derived Cell Apoptosis via the Protein Kinase C δ Pathway. *PLoS ONE* **2014**, *9*, e96089. [CrossRef] [PubMed]
24. Sano, R.; Reed, J.C. ER stress-induced cell death mechanisms. *Biochim. Biophys. Acta* **2013**, *1833*, 3460–3470. [CrossRef] [PubMed]
25. Roux, P.P.; Blenis, J. ERK and p38 MAPK-activated protein kinases: A family of protein kinases with diverse biological functions. *Microbiol. Mol. Biol. Rev.* **2004**, *68*, 320–344. [CrossRef] [PubMed]
26. Zhang, Y.; Xu, M.; Zhang, S.; Yan, L.; Yang, C.; Lu, W.; Li, Y.; Cheng, H. The role of G protein-coupled receptor 40 in lipoapoptosis in mouse β-cell line NIT-1. *Mol. Cell. Endocrinol.* **2007**, *38*, 651–661. [CrossRef] [PubMed]
27. Quan, X.; Zhang, L.; Li, Y.; Liang, C. TCF2 attenuates FFA-induced damage in islet β-cells by regulating production of insulin and ROS. *Int. J. Mol. Sci.* **2014**, *15*, 13317–13332. [CrossRef] [PubMed]
28. Wrede, C.E.; Dickson, L.M.; Lingohr, M.K.; Briaud, I.; Rhodes, C.J. Fatty acid and phorbol ester-mediated interference of mitogenic signaling via novel protein kinase C isoforms in pancreatic β-cells (INS-1). *J. Mol. Endocrinol.* **2003**, *30*, 271–286. [CrossRef] [PubMed]
29. Kyriakis, J.M.; Avruch, J. pp54 Microtubule-associated protein 2 kinase: A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *J. Biol. Chem.* **1990**, *265*, 17355–17363. [PubMed]

30. Kyriakis, J.M.; Brautigan, D.L.; Ingebritsen, T.S.; Avruch, J. pp54 microtubuleassociated protein-2 kinase requires both tyrosine and threonine phosphorylation for activity. *J. Biol. Chem.* **1991**, *266*, 10043–10046. [PubMed]
31. Derijard, B.; Hibi, M.; Wu, I.-H.; Barrett, T.; Su, B.; Deng, T.; Karin, M.; Davis, R.J. JNK1: A protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **1994**, *76*, 1025–1037. [CrossRef]
32. Kyriakis, J.M.; Banerjee, P.; Nikolakaki, E.; Dai, T.; Rubie, E.A.; Ahmad, M.F.; Avruch, J.; Woodgett, J.R. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* **1994**, *369*, 156–160. [CrossRef] [PubMed]
33. Kallunki, T.; Su, B.; Tsigelny, I.; Sluss, H.K.; Derijard, B.; Moore, G.; Davis, R.; Karin, M. JNK2 contains a specificity-determining region responsible for efficient C-Jun binding and phosphorylation. *Genes Dev.* **1994**, *8*, 2996–3007. [CrossRef]
34. Davis, R.J. Signal transduction by the JNK group of MAP kinases. *Cell* **2000**, *103*, 239–252. [CrossRef]
35. Hetz, C.; Martinon, F.; Rodriguez, D.; Glimcher, L.H. The unfolded protein response: Integrating stress signals through the stress sensor IRE1α. *Physiol. Rev.* **2011**, *91*, 1219–1243. [CrossRef] [PubMed]
36. Bogoyevitch, M.A.; Kobe, B. Uses for JNK: The many and varied substrates of the c-Jun N-terminal kinases. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 1061–1095. [CrossRef] [PubMed]
37. Natalicchio, A.; Labarbuta, R.; Tortosa, F.; Biondi, G.; Marrano, N.; Peschechera, A.; Carchia, E.; Orlando, M.R.; Leonardini, A.; Cignarelli, A.; et al. Exendin-4 protects pancreatic β cells from palmitate-induced apoptosis by interfering with GPR40 and the MKK4/7 stress kinase signalling pathway. *Diabetologia* **2013**, *56*, 2456–2466. [CrossRef] [PubMed]
38. Bachar, E.; Ariav, Y.; Ketzinel-Gilad, M.; Cerasi, E.; Kaiser, N.; Leibowitz, G. Glucose amplifies fatty acid-induced endoplasmic reticulum stress in pancreatic β-cells via activation of mTORC1. *PLoS ONE* **2009**, *4*, e4954. [CrossRef] [PubMed]
39. Cunha, D.A.; Hekerman, P.; Ladrière, L.; Bazarra-Castro, A.; Ortis, F.; Wakeham, M.C.; Moore, F.; Rasschaert, J.; Cardozo, A.K.; Bellomo, E.; et al. Initiation and execution of lipotoxic ER stress in pancreatic β-cells. *J. Cell Sci.* **2008**, *121*, 2308–2318. [CrossRef] [PubMed]
40. Cunha, D.A.; Igoillo-Esteve, M.; Gurzov, E.N.; Germano, C.M.; Naamane, N.; Marhfour, I.; Fukaya, M.; Vanderwinden, J.-M.; Gysemans, C.; Mathieu, C.; et al. Death protein 5 and p53-upregulated modulator of apoptosis mediate the endoplasmic reticulum stress—mitochondrial dialog triggering lipotoxic rodent and human β-cell apoptosis. *Diabetes* **2012**, *61*, 2763–2775. [CrossRef] [PubMed]
41. Oh, Y.S.; Lee, Y.J.; Kang, Y.; Han, J.; Lim, O.K.; Jun, H.S. Exendin-4 inhibits glucolipotoxic ER stress in pancreatic β cells via regulation of SREBP1c and C/EBPβ transcription factors. *J. Endocrinol.* **2013**, *216*, 343–352. [CrossRef] [PubMed]
42. Prause, M.; Christensen, D.P.; Billestrup, N.; Mandrup-Poulsen, T. JNK1 protects against glucolipotoxicity-mediated β-cell apoptosis. *PLoS ONE* **2014**, *9*, e87067.
43. Abaraviciene, S.M.; Lundquist, I.; Salehi, A. Rosiglitazone counteracts palmitate-induced β-cell dysfunction by suppression of MAP kinase, inducible nitric oxide synthase and caspase 3 activities. *Cell. Mol. Life Sci.* **2008**, *65*, 2256–2265. [CrossRef] [PubMed]
44. Kim, K.; Kim, D.-H.; Kim, Y.H. Compound K protects MIN6N8 pancreatic β-cells against palmitate-induced apoptosis through modulating SAPK/JNK activation. *Cell Biol. Int.* **2010**, *34*, 75–80. [CrossRef] [PubMed]
45. Aikin, R.; Maysinger, D.; Rosenberg, L. Cross-talk between phosphatidylinositol 3-kinase/AKT and c-jun NH2-terminal kinase mediates survival of isolated human islets. *Endocrinology* **2004**, *145*, 4522–4531. [CrossRef] [PubMed]
46. Nemcova-Fürstova, V.; Balusikova, K.; Sramek, J.; James, R.F.; Kovar, J. Caspase-2 and JNK activated by saturated fatty acids are not involved in apoptosis induction but modulate ER stress in human pancreatic β-cells. *Cell. Physiol. Biochem.* **2013**, *31*, 277–289.
47. Komiya, K.; Uchida, T.; Ueno, T.; Koike, M.; Abe, H.; Hirose, T.; Kawamori, R.; Uchiyama, Y.; Kominami, E.; Fujitani, Y.; et al. Free fatty acids stimulate autophagy in pancreatic β-cells via JNK pathway. *Biochem. Biophys. Res. Commun.* **2010**, *401*, 561–567. [CrossRef] [PubMed]
48. Akazawa, Y.; Cazanave, S.; Mott, J.L.; Elmi, N.; Bronk, S.F.; Kohno, S.; Charlton, M.R.; Gores, G.J. Palmitoleate attenuates palmitate-induced Bim and PUMA up-regulation and hepatocyte lipoapoptosis. *J. Hepatol.* **2010**, *52*, 586–593. [CrossRef] [PubMed]

49. Gunaratnam, K.; Vidal, C.; Boadle, R.; Thekkedam, C.; Duque, G. Mechanisms of palmitate-induced cell death in human osteoblasts. *Biol. Open* **2013**, *2*, 1382–1389. [CrossRef] [PubMed]
50. Win, S.; Than, T.A.; Le, B.H.; Garcia-Ruiz, C.; Fernandez-Checa, J.C.; Kaplowitz, N. Sab (Sh3bp5) dependence of JNK mediated inhibition of mitochondrial respiration in palmitic acid induced hepatocyte lipotoxicity. *J. Hepatol.* **2015**, *62*, 1367–1374. [CrossRef] [PubMed]
51. Mellor, H.; Parker, P.J. The extended protein kinase C superfamily. *Biochem. J.* **1998**, *332*, 281–292. [CrossRef] [PubMed]
52. Nishizuka, Y. Intracellular signaling by hydrolysis of phospholipids and activation of PKC. *Science* **1992**, *258*, 607–614. [CrossRef] [PubMed]
53. Kanashiro, C.A.; Khalil, R.A. Signal transduction by protein kinase C in mammalian cells. *Clin. Exp. Pharmacol. Physiol.* **1998**, *25*, 974–985. [CrossRef] [PubMed]
54. Reyland, M.E. Protein kinase Cdelta and apoptosis. *Biochem. Soc. Trans.* **2007**, *35*, 1001–1004. [CrossRef] [PubMed]
55. Zhao, M.; Xia, L.; Chen, G.Q. Protein kinase C δ in apoptosis: A brief overview. *Arch. Immunol. Ther. Exp.* **2012**, *60*, 361–372. [CrossRef] [PubMed]
56. Murriel, C.L.; Churchill, E.; Inagaki, K.; Szweda, L.I.; Mochly-Rosen, D. Protein kinase C delta activation induces apoptosis in response to cardiac ischemia and reperfusion damage: A mechanism involving BAD and the mitochondria. *J. Biol. Chem.* **2004**, *279*, 47985–47991. [CrossRef] [PubMed]
57. Lee, J.; Hong, F.; Kwon, S.; Kim, S.S.; Kim, D.O.; Kang, H.S.; Lee, S.J.; Ha, J.; Kim, S.S. Activation of p38MAPK induces cell cycle arrest via inhibition of C-Raf/ERK pathway during muscle differentiation. *Biochem. Biophys. Res. Commun.* **2002**, *298*, 765–771. [CrossRef]
58. Efimova, T.; Broome, A.M.; Eckert, R.L. Protein kinase C delta regulates keratinocyte death and survival by regulating activity and subcellular localization of a p38delta-extracellular signal regulated kinase 1/2 complex. *Mol. Cell. Biol.* **2004**, *24*, 8167–8183. [CrossRef] [PubMed]
59. Eitel, K.; Staiger, H.; Rieger, J.; Mischak, H.; Brandhorst, H.; Brendel, M.D.; Bretzel, R.G.; Häring, H.U.; Kellerer, M. Protein kinase C delta activation and translocation to the nucleus are required for fatty acid-induced apoptosis of insulin-secreting cells. *Diabetes* **2003**, *52*, 991–997. [CrossRef] [PubMed]
60. Simon, M.N.; Azevedo-Martins, A.K.; Amanso, A.M.; Carvalho, C.R.O.; Curi, R. Persistent activation of Akt or ERK prevents the toxicity induced by saturated and polyunsaturated fatty acids in RINm5F β-cells. *Toxicol. In Vitro* **2008**, *22*, 1018–1024. [CrossRef] [PubMed]
61. Alcáazar, O.; Qiu-yue, Z.; Gine, E.; Tamarit-Rodriguez, J. Stimulation of islet protein kinase C translocation by palmitate requires metabolism of the fatty acid. *Diabetes* **1997**, *46*, 1153–1158. [CrossRef]
62. Carpenter, L.; Cordery, D.; Biden, T.J. Inhibition of protein kinase C protects rat INS-1 cells against interleukin-1β and streptozotocin-induced apoptosis. *Diabetes* **2002**, *51*, 317–324. [CrossRef] [PubMed]
63. Denning, M.F.; Wang, Y.; Nickoloff, B.J.; Wrone-Smith, T. Protein kinase C δ is activated by caspase-dependent proteolysis during ultraviolet radiation-induced apoptosis of human keratinocytes. *J. Biol. Chem.* **1998**, *273*, 29995–30002. [CrossRef] [PubMed]
64. Reyland, M.E.; Anderson, S.M.; Matassa, A.A.; Barzen, K.A.; Quissell, D.O. Protein kinase C delta is essential for etoposide-induced apoptosis in salivary gland acinar cells. *J. Biol. Chem.* **1999**, *274*, 19115–19123. [CrossRef] [PubMed]
65. Choi, B.H.; Hur, E.M.; Lee, J.H.; Jun, D.J.; Kim, K.T. Protein kinase C δ-mediated proteasomal degradation of MAP kinase phosphatase-1 contributes to glutamate-induced neuronal cell death. *J. Cell Sci.* **2006**, *119*, 1329–1340. [CrossRef] [PubMed]
66. Jiang, Y.; Chen, C.; Li, Z.; Guo, W.; Gegner, J.A.; Lin, S.; Han, J. Characterization of the structure and function of a new mitogen-activated protein kinase (p38b). *J. Biol. Chem.* **1996**, *271*, 17920–17926. [CrossRef] [PubMed]
67. Lechner, C.; Zahalka, M.A.; Giot, J.F.; Moler, N.P.H.; Ullrich, A. ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 4355–4359. [CrossRef] [PubMed]
68. Li, Z.; Jiang, Y.; Ulevitch, R.J.; Han, J. The primary structure of p38γ: A new member of p38 group of MAP kinase. *Biochem. Biophys. Res. Commun.* **1996**, *228*, 334–340. [CrossRef] [PubMed]
69. Jiang, Y.; Gram, H.; Zhao, M.; New, L.; Gu, J.; Feng, L.; Di Padova, F.; Ulevitch, R.J.; Han, J. Characterization of the structure and function of the fourth member of p38 group mitogen activated protein kinases, p38δ. *J. Biol. Chem.* **1997**, *272*, 30122–30128. [CrossRef] [PubMed]

70. Kumar, S.; McDonnell, P.C.; Gum, R.J.; Hand, A.T.; Lee, J.C.; Young, P.R. Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochem. Biophys. Res. Commun.* **1997**, *235*, 533–538. [CrossRef] [PubMed]
71. Moriguchi, T.; Kuroyanagi, N.; Yamaguchi, K.; Gotoh, Y.; Irie, K.; Kano, T.; Shirakabe, K.; Muro, Y.; Shibuya, H.; Matsumoto, K.; et al. A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J. Biol. Chem.* **1996**, *271*, 13675–13679. [PubMed]
72. Ichijo, H.; Nishida, E.; Irie, K.; ten Dijke, P.; Saitoh, M.; Moriguchi, T.; Takagi, M.; Matsumoto, K.; Miyazono, K.; Gotoh, Y. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* **1997**, *275*, 90–94. [CrossRef] [PubMed]
73. Zhang, S.; Han, J.; Sells, M.A.; Chernoff, J.; Knaus, U.G.; Ulevitch, R.J.; Bokoch, G.M. Rho family GTPases regulate p38 MAP kinase through the downstream mediator Pak1. *J. Biol. Chem.* **1995**, *270*, 23934–23936. [CrossRef] [PubMed]
74. Cuenda, A.; Dorow, D.S. Differential activation of stress-activated protein kinase kinases SKK4/MKK7 and SKK1/MKK4 by the mixed-lineage kinase-2 and mitogen-activated protein kinase kinase (MKK) kinase-1. *Biochem. J.* **1998**, *333*, 11–15. [CrossRef] [PubMed]
75. Takekawa, M.; Posas, F.; Saito, H. A human homolog of the yeast Ssk2/Ssk22 MAP kinase kinase kinases, MTK1, mediates stress induced activation of the p38 and JNK pathways. *EMBO J.* **1997**, *16*, 4973–4982. [CrossRef] [PubMed]
76. Bagrodia, S.; Derijard, B.; Davis, R.J.; Cerione, R.A. Cdc42 and PAK mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. *J. Biol. Chem.* **1995**, *270*, 27995–27998. [PubMed]
77. Zarubin, T.; Han, J. Activation and signaling of the p38 MAP kinase pathway. *Cell Res.* **2005**, *15*, 11–18. [CrossRef] [PubMed]
78. Cuenda, A.; Rousseau, S. p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim. Biophys. Acta* **2004**, *1773*, 1358–1375. [CrossRef] [PubMed]
79. Liu, J.; Chang, F.; Li, F.; Fu, H.; Wang, J.; Zhang, S.; Zhao, J.; Yin, D. Palmitate promotes autophagy and apoptosis through ROS-dependent JNK and p38 MAPK. *Biochem. Biophys. Res. Commun.* **2015**, *463*, 262–267. [CrossRef] [PubMed]
80. Sramek, J.; Nemcova-Fürstova, V.; Balusikova, K.; Daniel, P.; Jelinek, M.; James, R.F.; Kovar, J. p38 MAPK is activated but does not play a key role during apoptosis induction by saturated fatty acid in human pancreatic β-cells. *Int. J. Mol. Sci.* **2016**, *17*, 159. [CrossRef] [PubMed]
81. Yang, G.; Yang, W.; Wu, L.; Wang, R. H2S, endoplasmic reticulum stress, and apoptosis of insulin-secreting β cells. *J. Biol. Chem.* **2007**, *282*, 16567–16576. [CrossRef] [PubMed]
82. Ma, K.; Nunemaker, C.S.; Wu, R.; Chakrabarti, S.K.; Taylor-Fishwick, D.A.; Nadler, J.L. 12-Lipoxygenase products reduce insulin secretion and β-cell viability in human islets. *J. Clin. Endocrinol. Metab.* **2010**, *95*, 887–893. [CrossRef] [PubMed]
83. Makeeva, N.; Myers, J.W.; Welsh, N. Role of MKK3 and p38 MAPK in cytokine-induced death of insulin-producing cells. *Biochem. J.* **2006**, *393*, 129–139. [CrossRef] [PubMed]
84. Saldeen, J.; Lee, J.C.; Welsh, N. Role of p38 mitogen-activated protein kinase (p38 MAPK) in cytokine-induced rat islet cell apoptosis. *Biochem. Pharmacol.* **2001**, *61*, 1561–1569. [CrossRef]
85. Chai, W.; Liu, Z. p38 mitogen-activated protein kinase mediates palmitate-induced apoptosis but not inhibitor of nuclear factor-kappa B degradation in human coronary artery endothelial cells. *Endocrinology* **2007**, *148*, 1622–1628. [CrossRef] [PubMed]
86. Liu, Z.; Cao, W. p38 mitogen-activated protein kinase: a critical node linking insulin resistance and cardiovascular diseases in type 2 diabetes mellitus. *Endocr. Metab. Immune Disord. Drug Targets* **2009**, *9*, 38–46. [CrossRef] [PubMed]
87. Song, Y.; Li, X.; Li, Y.; Li, N.; Shi, X.; Ding, H.; Zhang, Y.; Li, X.; Liu, G.; Wang, Z. Non-esterified fatty acids activate the ROS-p38-p53/Nrf2 signaling pathway to induce bovine hepatocyte apoptosis in vitro. *Apoptosis* **2014**, *19*, 984–997. [CrossRef] [PubMed]
88. Bramanti, V.; Grasso, S.; Tibullo, D.; Giallongo, C.; Raciti, G.; Viola, M.; Avola, R. Modulation of extracellular signal-related kinase, cyclin D1, glial fibrillary acidic protein, and vimentin expression in estradiol-pretreated astrocyte cultures treated with competence and progression growth factors. *J. Neurosci. Res.* **2015**, *93*, 1378–1387. [CrossRef] [PubMed]

89. Gudermann, T. Multiple pathways of ERK activation by G protein-coupled receptors. *Novartis Found. Symp.* **2001**, *239*, 68–79. [PubMed]
90. Chang, F.; Steelman, L.S.; Lee, J.T.; Shelton, J.G.; Navolanic, P.M.; Blalock, W.L.; Franklin, R.A.; McCubrey, J.A. Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: Potential targeting for therapeutic intervention. *Leukemia* **2003**, *17*, 1263–1293. [CrossRef] [PubMed]
91. Kolch, W.; Heidecker, G.; Kochs, G.; Hummel, R.; Vahidi, H.; Mischak, H.; Finkenzeller, G.; Marme, D.; Rapp, U.R. Protein kinase Ca activates RAF-1 by direct phosphorylation. *Nature* **1993**, *364*, 249–252. [CrossRef] [PubMed]
92. Fabian, J.R.; Daar, I.O.; Morrison, D.K. Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. *Mol. Cell. Biol.* **1993**, *13*, 7170–7179. [CrossRef] [PubMed]
93. Diaz, B.; Barnard, D.; Filson, A.; MacDonald, S.; King, A.; Marshall, M. Phosphorylation of Raf-1 serine 338-serine 339 is an essential regulatory event for Ras-dependent activation and biological signaling. *Mol. Cell. Biol.* **1997**, *17*, 4509–4516. [CrossRef] [PubMed]
94. Chen, Z.; Gibson, T.B.; Robinson, F.; Silvestro, L.; Pearson, G.; Xu, B.; Wright, A.; Vanderbilt, C.; Cobb, M.H. MAP Kinases. *Chem. Rev.* **2001**, *101*, 2449–2476. [CrossRef] [PubMed]
95. Bramanti, V.; Grasso, S.; Tibullo, D.; Giallongo, C.; Pappa, R.; Brundo, M.V.; Tomassoni, D.; Viola, M.; Amenta, F.; Avola, R. Neuroactive molecules and growth factors modulate cytoskeletal protein expression during astroglial cell proliferation and differentiation in culture. *J. Neurosci. Res.* **2016**, *94*, 90–98. [CrossRef] [PubMed]
96. Cagnol, S.; Chambard, J.C. ERK and cell death: Mechanisms of ERK-induced cell death—Apoptosis, autophagy and senescence. *FEBS J.* **2010**, *277*, 2–21. [CrossRef] [PubMed]
97. Guo, J.; Qian, Y.Y.; Xi, X.X.; Hu, X.H.; Zhu, J.X.; Han, X. Blockage of ceramide metabolism exacerbates palmitate inhibition of pro-insulin gene expression in pancreatic β-cells. *Mol. Cell. Biochem.* **2010**, *338*, 283–290. [CrossRef] [PubMed]
98. Plaisance, V.; Perret, V.; Favrea, D.; Abderrahmania, A.; Yanga, J.-Y.; Widmanna, C.; Regazzi, R. Role of the transcriptional factor C/EBP in free fatty acid-elicited β cell failure. *Mol. Cell. Endocrinol.* **2009**, *305*, 47–55. [CrossRef] [PubMed]
99. Fontes, G.; Semache, M.; Hagman, D.K.; Tremblay, C.; Shah, R.; Rhodes, C.J.; Rutter, J.; Poitout, V. Involvement of Per-Arnt-Sim Kinase and extracellular-regulated kinases-1/2 in palmitate inhibition of insulin gene expression in pancreatic β-cells. *Diabetes* **2009**, *58*, 2048–2058. [CrossRef] [PubMed]
100. Watson, M.L.; Macrae, K.; Marley, A.E.; Hundal, H.S. Chronic effects of palmitate overload on nutrient-induced insulin secretion and autocrine signalling in pancreatic MIN6 β cells. *PLoS ONE* **2011**, *6*, e25975. [CrossRef] [PubMed]
101. Larsen, C.M.; Wadt, K.A.; Juhl, L.F.; Andersen, H.U.; Karlsen, A.E.; Su, M.S.; Seedorf, K.; Shapiro, L.; Dinarello, C.A.; Mandrup-Poulsen, T. Interleukin-1β-induced rat pancreatic islet nitric oxide synthesis requires both the p38 and extracellular signal-regulated kinase 1/2 mitogen-activated protein kinases. *J. Biol. Chem.* **1998**, *273*, 15294–15300. [CrossRef] [PubMed]
102. Maedler, K.; Storling, J.; Sturis, J.; Zuellig, R.A.; Spinas, G.A.; Arkhammar, P.O.; Mandrup-Poulsen, T.; Donath, M.Y. Glucose- and interleukin-1β-induced β-cell apoptosis requires Ca^{2+} influx and extracellular signal-regulated kinase (ERK) 1/2 activation and is prevented by a sulfonylurea receptor 1/inwardly rectifying K^+ channel 6.2 (SUR/Kir6.2) selective potassium channel opener in human islets. *Diabetes* **2004**, *53*, 1706–1713. [PubMed]
103. Lawrence, M.C.; McGlynn, K.; Park, B.H.; Cobb, M.H. ERK1/2-dependent activation of transcription factors required for acute and chronic effects of glucose on the insulin gene promoter. *J. Biol. Chem.* **2005**, *280*, 26751–26759. [CrossRef] [PubMed]
104. Martino, L.; Masini, M.; Novelli, M.; Giacopelli, D.; Beffy, P.; Masiello, P.; de Tata, V. The aryl receptor inhibitor epigallocatechin-3-gallate protects INS-1E β-cell line against acute dioxin toxicity. *Chemosphere* **2013**, *93*, 1447–1455. [CrossRef] [PubMed]
105. Watanabe, S.; Wei, F.Y.; Matsunaga, T.; Matsunaga, N.; Kaitsuka, T.; Tomizawa, K. Oxytocin protects against stress-induced cell death in murine pancreatic β-cells. *Sci. Rep.* **2016**, *6*, 25185. [CrossRef] [PubMed]
106. Ahn, M.; Yoder, S.M.; Wang, Z.; Oh, E.; Ramalingam, L.; Tunduguru, R.; Thurmond, D.C. The p21-activated kinase (PAK1) is involved in diet-induced β cell mass expansion and survival in mice and human islets. *Diabetologia* **2016**. in press. [CrossRef] [PubMed]

107. Kim, J.E.; Ahn, M.W.; Baek, S.H.; Lee, I.K.; Kim, Y.W.; Kim, J.Y.; Dan, J.M.; Park, S.Y. AMPK activator, AICAR, inhibits palmitate-induced apoptosis in osteoblast. *Bone* **2008**, *43*, 394–404. [CrossRef] [PubMed]
108. Dong, X.; Bi, L.; He, S.; Meng, G.; Wei, B.; Jia, S.; Liu, J. FFAs-ROS-ERK/P38 pathway plays a key role in adipocyte lipotoxicity on osteoblasts in co-culture. *Biochimie* **2014**, *101*, 123–131. [CrossRef] [PubMed]
109. Shi, G.; Sun, C.; Gu, W.; Yang, M.; Zhang, X.; Zhai, N.; Lu, Y.; Zhang, Z.; Shou, P.; Zhang, Z.; Ning, G. Free fatty acid receptor 2, a candidate target for type 1 diabetes, induces cell apoptosis through ERK signaling. *J. Mol. Endocrinol.* **2014**, *53*, 367–380. [CrossRef] [PubMed]
110. Hanada, M.; Feng, J.; Hemmings, B.A. Structure, regulation and function of PKB/AKT—A major therapeutic target. *Biochim. Biophys. Acta* **2004**, *1697*, 3–16. [CrossRef] [PubMed]
111. Morales, B.M.; de Plata, A.C. Role of AKT/mTORC1 pathway in pancreatic β-cell proliferation. *Colomb. Med.* **2012**, *43*, 235–243.
112. Olsson, A.K.; Dimberg, A.; Kreuger, J.; Claesson-Welsh, L. VEGF receptor signalling—In control of vascular function. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 359–371. [CrossRef] [PubMed]
113. Kim, S.-J.; Winter, K.; Nian, C.; Tsuneoka, M.; Koda, Y.; McIntosh, C.H.S. Glucose-dependent insulinotropic polypeptide (GIP) stimulation of pancreatic B cell survival is dependent upon phosphatidylinositol3-kinase (PI3K)/protein kinase B (PKB) signaling, inactivation of the forkhead transcription factor FoxO1, and down-regulation of bax expression. *J. Biol. Chem.* **2005**, *280*, 22297–22307. [PubMed]
114. Del Peso, L.; Gonzalez-Garcia, M.; Page, C.; Herrera, R.; Nunez, G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* **1997**, *278*, 687–689. [CrossRef] [PubMed]
115. Ozes, O.N.; Mayo, L.D.; Gustin, J.A.; Pfeffer, S.R.; Pfeffer, L.M.; Donner, D.B. NF-κB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* **1999**, *401*, 82–85. [CrossRef] [PubMed]
116. Nicoletti-Carvalho, J.E.; Nogueira, T.C.; Gorjão, R.; Bromati, C.R.; Yamanaka, T.S.; Boschero, A.C.; Velloso, L.A.; Curi, R.; Anhê, G.F.; Bordin, S. UPR-mediated TRIB3 expression correlates with reduced AKT phosphorylation and inability of interleukin 6 to overcome palmitate-induced apoptosis in RINm5F cells. *J. Endocrinol.* **2010**, *206*, 183–193. [CrossRef] [PubMed]
117. Li, X.J.; Guo, Q.H.; Wang, X.; Xue, B.; Sun, L.Q.; Meng, Q.T.; Lu, J.M.; Mu, Y.M. LRP16 gene protects mouse insulinoma MIN6 cells against fatty acid-induced apoptosis through Akt/FoxO1 signaling. *Chin. Med. J.* **2012**, *125*, 1695–1702. [PubMed]
118. Shao, S.; Nie, M.; Chen, C.; Chen, X.; Zhang, M.; Yuan, G.; Yu, X.; Yang, Y. Protective action of liraglutide in β cells under lipotoxic stress via PI3K/Akt/FoxO1 pathway. *J. Cell. Biochem.* **2014**, *115*, 1166–1175. [CrossRef] [PubMed]
119. Hao, F.; Kang, J.; Cao, Y.; Fan, S.; Yang, H.; An, Y.; Pan, Y.; Tie, L.; Li, X. Curcumin attenuates palmitate-induced apoptosis in MIN6 pancreatic β-cells through PI3K/Akt/FoxO1 and mitochondrial survival pathways. *Apoptosis* **2015**, *20*, 1420–1432. [CrossRef] [PubMed]
120. Qin, L.; Wang, Z.; Tao, L.; Wang, Y. ER stress negatively regulates AKT/TSC/mTOR pathway to enhance autophagy. *Autophagy* **2010**, *6*, 239–247. [CrossRef] [PubMed]
121. Nakajima, S.; Hiramatsu, N.; Hayakawa, K.; Saito, Y.; Kato, H.; Huang, T.; Yao, J.; Paton, A.W.; Paton, J.C.; Kitamura, M. Selective abrogation of BiP/GRP78 blunts activation of NF-κB through the ATF6 branch of the UPR: Involvement of C/EBPβ and mTOR-dependent dephosphorylation of Akt. *Mol. Cell. Biol.* **2011**, *31*, 1710–1718. [CrossRef] [PubMed]
122. Tuttle, R.L.; Gill, N.S.; Pugh, W.; Lee, J.P.; Koeberlein, B.; Furth, E.E.; Polonsky, K.S.; Naji, A.; Birnbaum, M.J. Regulation of pancreatic β-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. *Nat. Med.* **2001**, *7*, 1133–1137. [CrossRef] [PubMed]
123. Kim, N.; Jeong, S.; Jing, K.; Shin, S.; Kim, S.; Heo, J.Y.; Kweon, G.R.; Park, S.K.; Wu, T.; Park, J.I.; et al. Docosahexaenoic acid induces cell death in human non-small cell lung cancer cells by repressing mTOR via AMPK activation and PI3K/Akt inhibition. *BioMed Res. Int.* **2015**, *2015*, 239764. [CrossRef] [PubMed]
124. Su, J.; Zhou, H.; Tao, Y.; Guo, J.; Guo, Z.; Zhang, S.; Zhang, Y.; Huang, Y.; Tang, Y.; Dong, Q.; et al. G-CSF protects human brain vascular endothelial cells injury induced by high glucose, free fatty acids and hypoxia through MAPK and Akt signaling. *PLoS ONE* **2015**, *10*, e0120707.

7.4. PUBLIKACE 4

Šrámek J, Němcová-Fürstová V, Kovář J:
Effect of saturated stearic acid on MAP kinase and ER stress signaling pathways during apoptosis induction in human pancreatic β-cells is inhibited by unsaturated oleic acid.
International Journal of Molecular Sciences, 18: 2313, 2017.

International Journal of *Molecular Sciences*

MDPI

Communication

Effect of Saturated Stearic Acid on MAP Kinase and ER Stress Signaling Pathways during Apoptosis Induction in Human Pancreatic β-Cells Is Inhibited by Unsaturated Oleic Acid

Jan Šrámek, Vlasta Němcová-Fürstová, Nela Pavlíková and Jan Kovář *

Division of Cell and Molecular Biology & Center for Research of Diabetes, Metabolism and Nutrition, Third Faculty of Medicine, Charles University, Ruská 87, 100 00 Prague, Czech Republic; jan.sramek@lf3.cuni.cz (J.Š.); vlasta.furstova@centrum.cz (V.N.-F.); nela.pavlikova@lf3.cuni.cz (N.P.)
* Correspondence: jan.kovar@lf3.cuni.cz; Tel.: +420-267-102-658

Received: 27 September 2017; Accepted: 31 October 2017; Published: 2 November 2017

Abstract: It has been shown that saturated fatty acids (FAs) have a detrimental effect on pancreatic β-cells function and survival, leading to apoptosis, whereas unsaturated FAs are well tolerated and are even capable of inhibiting the pro-apoptotic effect of saturated FAs. Molecular mechanisms of apoptosis induction and regulation by FAs in β-cells remain unclear; however, mitogen-activated protein (MAP) kinase and endoplasmic reticulum (ER) stress signaling pathways may be involved. In this study, we tested how unsaturated oleic acid (OA) affects the effect of saturated stearic acid (SA) on the p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathways as well as the ER stress signaling pathways during apoptosis induction in the human pancreatic β-cells NES2Y. We demonstrated that OA is able to inhibit all effects of SA. OA alone has only minimal or no effects on tested signaling in NES2Y cells. The point of OA inhibitory intervention in SA-induced apoptotic signaling thus seems to be located upstream of the discussed signaling pathways.

Keywords: fatty acids; pancreatic β-cells; apoptosis; p38 mitogen-activated protein kinase (MAPK); extracellular signal-regulated kinase (ERK); endoplasmic reticulum (ER) stress; NES2Y

1. Introduction

Increased levels of fatty acids (FAs) in blood are considered to be one of the main factors responsible for pancreatic β-cell death in type 2 diabetes [1–5]. Our previous studies as well as other studies have shown that this effect of FAs is related to the saturation of particular FAs. Saturated FAs (palmitic or stearic acid) have detrimental effects on pancreatic β-cells survival, leading to apoptosis, whereas unsaturated FAs (oleic or palmitoleic acid) are well tolerated and even capable of inhibiting the pro-apoptotic effect of saturated FAs [2,4–8]. Molecular mechanisms of apoptosis induction and its regulation by FAs in β-cells remain unclear [9]. However, it was shown that apoptosis induced by saturated FAs can be mediated by p38 MAPK (mitogen-activated protein kinase) and ERK (extracellular signal-regulated kinases) MAPK signaling pathways [10–14] as well as by endoplasmic reticulum (ER) stress signaling [15,16].

The p38 MAPK pathway becomes mainly activated by the dual-specific MKK3/6 (mitogen-activated protein kinase kinase 3 and 6) kinase due to various extracellular stimuli such as chemical stresses (reviewed in [17]). p38 MAPK regulates the activity of MAPKAPK-2 (MAPK-activated protein kinase 2) which is involved in nuclear export of activated p38 MAPK [18]. It may also affect the activation of some proteins such as NF-κB (nuclear factor kappa B) [19] or caspase-3 [20]. These proteins are involved in the regulation of apoptosis induction.

The ERK pathway is mostly activated by growth factors. Growth factors, acting through receptor kinases and adaptor protein son of sevenless (SOS), activate Ras GTPase, which is responsible for c-Raf phosphorylation [21]. This kinase consequently phosphorylates MEK1/2 (mitogen-activated protein kinase/ERK kinase), which leads to the phosphorylation and thus activation of ERK1/2 kinase. Like p38 MAPK, ERK1/2 can affect the activation of various proteins that also regulate apoptosis, such as Fox03a (forkhead box O3a) or several proteins of the Bcl-2 (B-cell lymphoma) family [22].

ER stress signaling is represented by three known pathways. Their activation seems to begin on the ER membrane by the activation of three proteins: (1) IRE1α (inositol-requiring protein 1α); (2) PERK (protein kinase RNA (PKR)-like ER kinase); and (3) ATF6 (activating transcription factor 6). Activated IRE1α causes unconventional splicing of mRNA for XBP1 (X-box binding protein 1), which leads to the translation of active transcription factor (XBP1s). It also leads to JNK (c-Jun N-terminal kinase) activation by phosphorylation, which further phosphorylates c-Jun. Activated PERK results in the inhibition of protein translation via eIF2α (eukaryotic initiation factor 2α) phosphorylation. ATF6 translocation to the nucleus, where ATF6 functions as a transcription factor, represents the activation of the ATF6 pathway. The main purpose of these signaling patterns is to restore ER homeostasis, e.g., by decreasing protein translation and increasing the expression of chaperones, such as the ER chaperone BiP (immunoglobulin heavy chain-binding protein) [23]. However, failure of these responses leads to apoptosis induction by not fully clear mechanisms. The proposed mediator is transcription factor CHOP (CCAAT-enhancer-binding protein (C/EBP) homologous protein) [24].

In the present study, we tested how unsaturated OA affects the effect of saturated SA on the p38 MAPK and ERK pathways as well as the ER stress signaling pathways during apoptosis induction in the human pancreatic β-cells NES2Y. We demonstrate that OA is able to inhibit all effects of SA. OA alone had minimal or no effects on tested signaling in NES2Y cells. The point of OA inhibitory intervention in SA-induced apoptotic signaling thus seems to be located upstream of the discussed signaling pathways.

2. Results

2.1. Effect of Oleic Acid on Stearic Acid-Induced Apoptosis

We showed that OA (0.2 mM) inhibited SA-induced (1 mM) cell death of NES2Y cells. OA applied alone has no or only minimal effects on NES2Y cells (Figure 1A). Cell death induced by SA is associated with the activation of initiator caspase-9 and -8 as well as executioner caspase-7 and poly ADP-ribose polymerase (PARP) (substrate of caspase-7) cleavage. Thus, it can be characterized as apoptosis. The activation of caspases as well as PARP cleavage is inhibited by the co-incubation of OA (Figure 1B,C). Cleavage of caspase-2 and -3 was not tested, since we found previously that there is nearly no activation of caspase-3 in NES2Y cells after SA exposure, and that caspase-2 does not play a key role in SA-induced apoptosis [8,25].

2.2. Effect of Oleic Acid on the Activation of the p38 MAPK and the Inhibition of the ERK Signaling Pathways by Stearic Acids

We previously showed the activation of the p38 MAPK signaling pathway and the inhibition of the ERK signaling pathway within 24 h of the application of apoptosis-inducing SA (1 mM) in NES2Y β-cells [14]. In order to test whether OA (0.2 mM) could interfere with the effect of SA, we assessed the activation of the p38 MAPK signaling pathway and inhibition of the ERK signaling pathway within 24 h after OA co-incubation with SA in NES2Y cells.

The effect of SA on the activation of the p38 pathway members, i.e., an increase in the level of phosphorylated MKK3/6, p38 MAPK, and MAPKAPK-2, was inhibited by co-incubation with OA as early as 12 h after the treatment in all of the tested proteins. OA applied alone resulted in low or nearly no changes in the level of phosphorylated p38 MAPK pathway members. No change was detected in the level of total p38 MAPK within 24 h of FA treatment (Figure 2A).

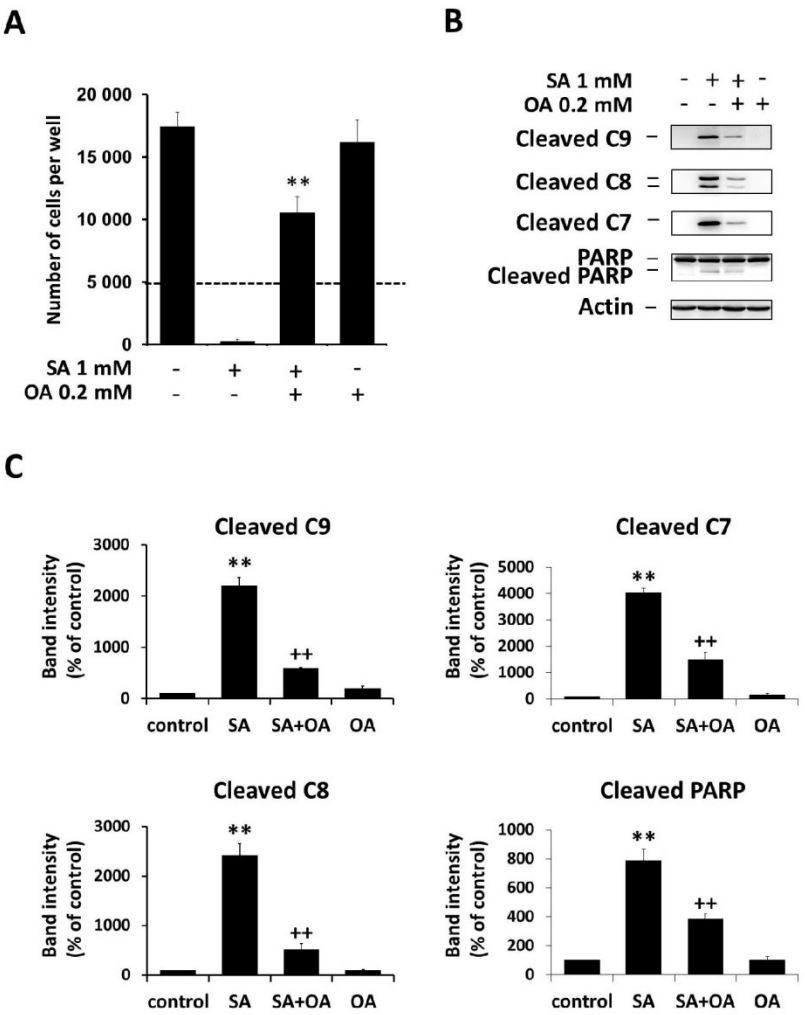


Figure 1. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied simultaneously with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid (see Section 4) on (**A**) cell growth and viability, (**B**) the level of cleaved caspase-9 (C9), caspase-8 (C8), caspase-7 (C7), and PARP in NES2Y cells. Cells incubated without fatty acids represented control cells. (**A**) Cells were seeded at a concentration of 5×10^3 cells/100 μL of culture medium per well of 96-well plate (see Section 4). The number of living cells was determined after 96 h of incubation. The number of cells of the inoculum is shown as a dashed line. Each column represents the mean of four separate cultures ± standard error of the mean (SEM). ** $p < 0.01$ when comparing the effect of 1 mM stearic acid applied together with 0.2 mM oleic acid and the effect of 1 mM stearic acid alone. (**B**) After 18 h of incubation (see Section 4), the levels of individual proteins were assessed using Western blot analysis and relevant antibodies (see Section 4). Actin was included to confirm equal protein loading. The data presented were obtained in one representative experiment from at least three independent experiments. (**C**) Densitometric analysis of data from Western blotting are also shown. Each column represents the mean of three experimental values ± SEM. ** $p < 0.01$ when comparing the effect of SA with control cells, ++ $p < 0.05$ when comparing the effect of SA plus OA with the effect of SA alone.

The effect of SA on the inhibition of the ERK pathway members, i.e., a decrease in the level of phosphorylated c-Raf, MEK1/2, and ERK1/2, was also inhibited by OA co-incubation within 24 h of the treatment. Separate application of OA resulted in nearly no effect on the level of phosphorylated

Int. J. Mol. Sci. **2017**, *18*, 2313

ERK pathway members. We did not detect any change in the level of total ERK1/2 within 24 h of FA treatment (Figure 2B).

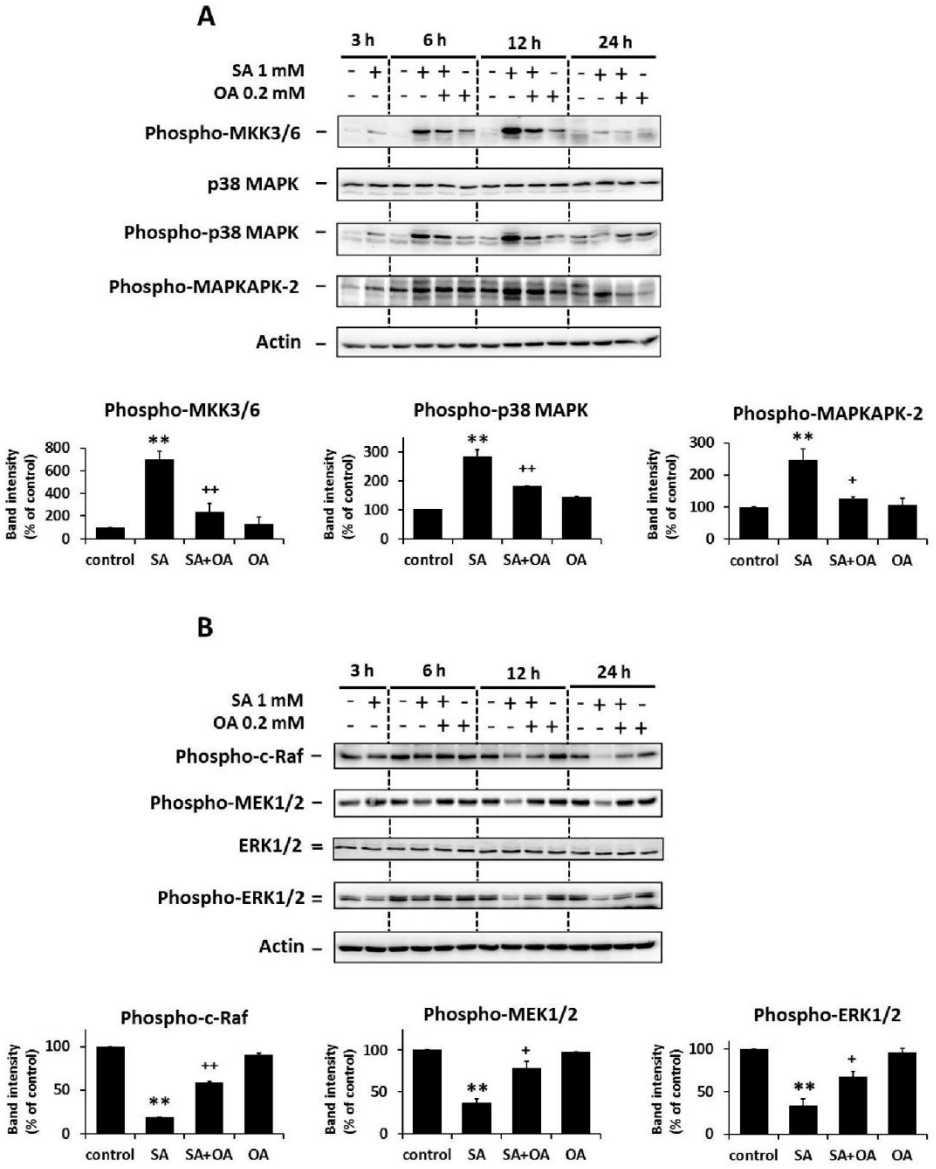


Figure 2. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied simultaneously with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid (see Section 4) on (**A**) the levels of phospho-MKK3/6, p38 MAPK, phospho-p38 MAPK, and phospho-MAPKAPK-2 (the p38 MAPK signaling pathway); and (**B**) the levels of phospho-c-Raf, phospho-MEK1/2, ERK1/2, and phospho-ERK1/2 (the ERK signaling pathway) in NES2Y cells. Cells incubated without fatty acids represented control cells. After 3, 6, 12, and 24 h of incubation (see Section 4), the levels of individual proteins were assessed using Western blot analysis and relevant antibodies (see Section 4). Actin was included to confirm equal protein loading. The data presented were obtained in one representative experiment from at least three independent experiments. Densitometric analysis of data from Western blotting is also shown. The analysis was carried out for 12 h after fatty acids application in the case of the p38 MAPK pathway and for 24 h in the case of the ERK pathway. Each column represents the mean of three experimental values ± SEM. ** $p < 0.01$ when comparing the effect of SA with control cells, $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$ when comparing the effect of SA plus OA with the effect of SA alone.

Int. J. Mol. Sci. **2017**, *18*, 2313

2.3. Effect of Oleic Acid on the Activation of the ER Stress Signaling Pathways by Stearic Acid

We previously documented the activation of ER stress signaling pathways (IRE1α and PERK pathways) within 24 h of apoptosis-inducing SA (1 mM) treatment in NES2Y β-cells [25]. In order to test whether OA (0.2 mM) could affect the SA-induced activation of ER stress signaling pathways, we assessed the activation of members of the ER stress IRE1α pathway (the levels of phospho-IRE1α, phospho-JNK, phospho-c-Jun) and the PERK pathway (the level of phospho-eIF2α) within 24 h after OA co-incubation with SA in NES2Y cells. The level of XBP1 splicing as well as the level of two downstream effector molecules, CHOP and BiP (known as ER stress markers), were also tested.

The effect of SA on the activation of ER stress pathways, i.e., an increase in the levels of phospho-IRE1α (and IRE1α), phospho-JNK (JNK level was not changed), phospho-c-Jun (and c-Jun), phospho-eIF2α (eIF2α level was not changed), CHOP and BiP, as well as the induction of XBP1 splicing, was significantly inhibited by OA co-incubation within 24 h of the treatment. Separate application of OA resulted in nearly no changes in the level of tested proteins and XBP1 splicing (Figures 3–5).

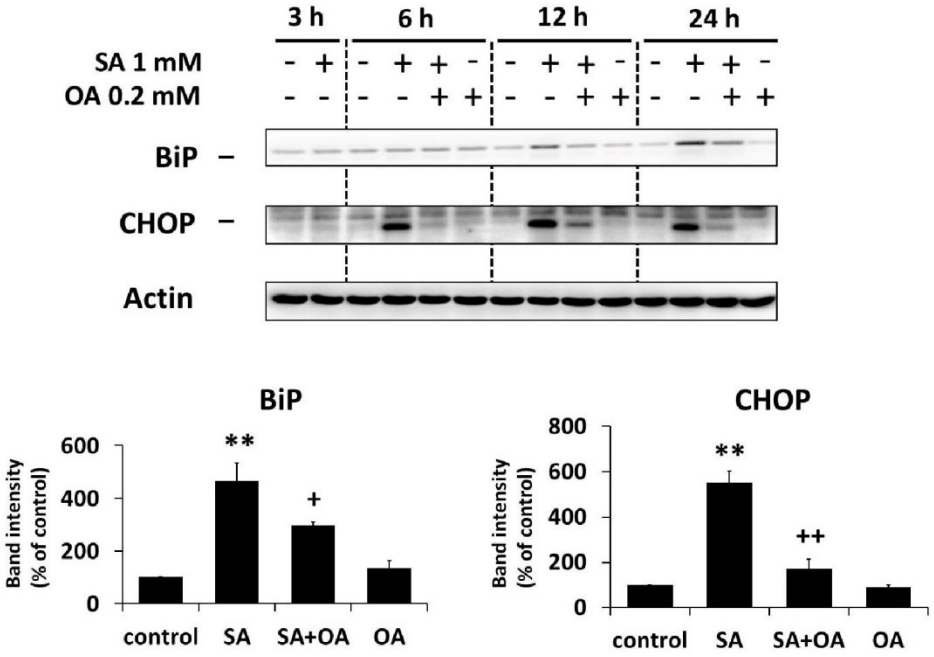


Figure 3. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied simultaneously with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid (see Section 4) on the level of ER stress markers BiP and CHOP in NES2Y cells. Cells incubated without fatty acids represented control cells. After 3, 6, 12, and 24 h of incubation (see Section 4), the levels of individual proteins were assessed using Western blot analysis and relevant antibodies (see Section 4). Actin was included to confirm equal protein loading. The data presented were obtained in one representative experiment from at least three independent experiments. Densitometric analysis of data from Western blotting is also shown. The analysis was carried out for 24 h after fatty acids application. Each column represents the mean of three experimental values ± SEM. ** $p < 0.01$ when comparing the effect of SA with control cells, $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$ when comparing the effect of SA plus OA with the effect of SA alone.

Int. J. Mol. Sci. **2017**, *18*, 2313

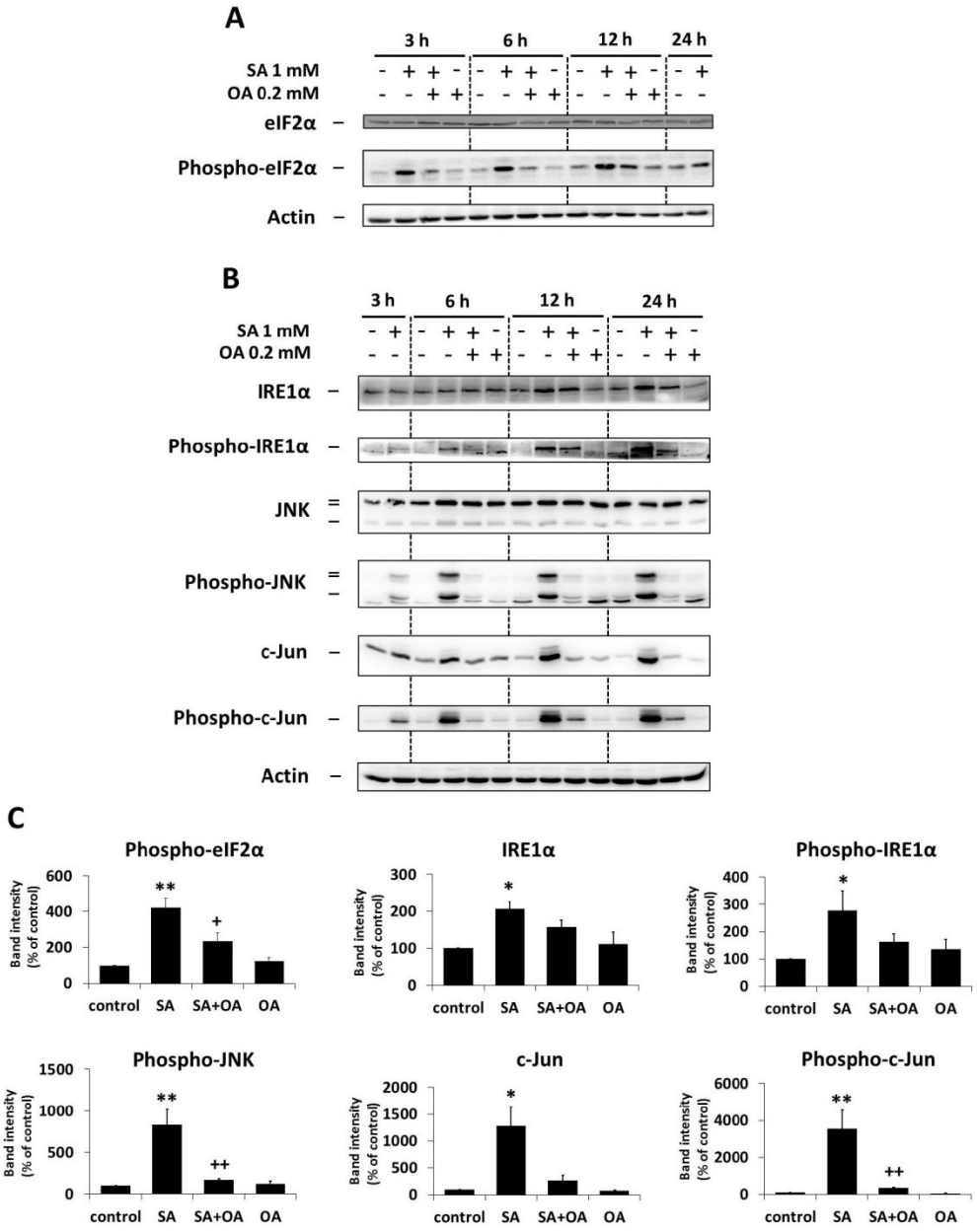


Figure 4. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied simultaneously with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid (see Section 4) on the levels of (**A**) eIF2α and phospho-eIF2α, and (**B**) IRE1α, phospho-IRE1α, JNK, phospho-JNK, c-Jun, and phospho-c-Jun in NES2Y cells. Cells incubated without fatty acids represented control cells. After 3, 6, 12, and 24 h of incubation (see Section 4) (**A**,**B**), the levels of individual proteins were assessed using Western blot analysis and relevant antibodies (see Section 4). Actin was included to confirm equal protein loading. (**C**) Densitometric analysis of data from Western blotting is also shown. The analysis was carried out for 6 h after fatty acids application in the case of phospho-eIF2α, for 12 h in the case of phospho-JNK, c-Jun and phospho-c-Jun, and for 24 h in the case of IRE1α and phospho-IRE1α. Each column represents the mean of three experimental values ± SEM. * $p < 0.05$, ** $p < 0.01$ when comparing the effect of SA with control cells. $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$ when comparing the effect of SA plus OA with the effect of SA alone.

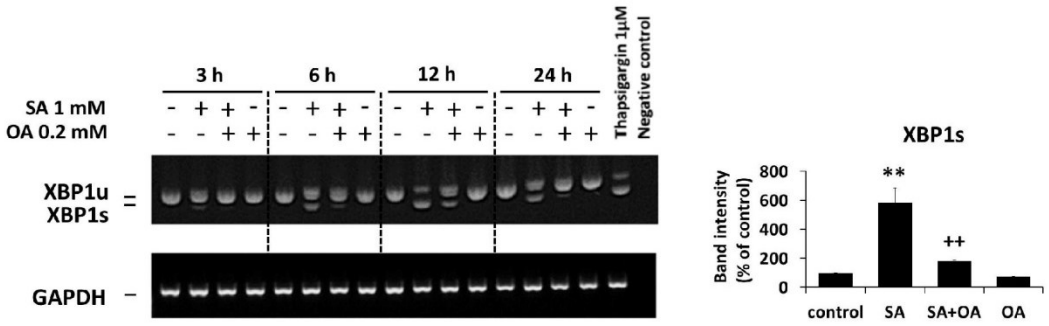


Figure 5. Effect of 1 mM stearic acid (SA), 1 mM stearic acid applied simultaneously with 0.2 mM oleic acid (OA), and 0.2 mM oleic acid (see Section 4) on XBP1 splicing in NES2Y cells. Cells incubated without fatty acids represented control cells. After 3, 6, 12, and 24 h of incubation (see Section 4), the XBP1 splicing was assessed by RT-PCR using relevant primers (see Section 4). NES2Y cells were treated with 1 μM thapsigargin as a positive control of XBP1 splicing. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a control gene for RT-PCR. The data presented were obtained in one representative experiment from at least three independent experiments. Densitometric analysis of data from RT-PCR is also shown. The analysis was carried out for 24 h after fatty acids application. Each column represents the mean of three experimental values ± SEM. ** $p < 0.01$ when comparing the effect of SA with control cells, $^{++}$ $p < 0.01$ when comparing the effect of SA plus OA with the effect of SA alone.

3. Discussion

We, as well as other authors, have shown that unsaturated FAs have nearly no detrimental effect on pancreatic β-cells, but that they are in fact capable of inhibiting the pro-apoptotic effect of saturated FAs [2,4–8]. The molecular mechanisms involved in the modulation of saturated FAs-induced apoptosis by unsaturated FAs in β-cells are not fully clear. However, it seems that the effect of unsaturated FAs is mediated by the regulation of some signaling pathways rather than by their direct interference with the metabolism of saturated FAs [6,26,27]. Therefore, in this paper we tested the modulation of the effects of apoptosis-inducing saturated SA on the p38 MAPK pathway, the ERK pathway, and also on ER stress signaling pathways IRE1α and PERK by unsaturated OA.

Our data showed that the activation of the p38 MAPK pathway, inhibition of the ERK pathway, and activation of the IRE1α and PERK ER stress pathways by SA were inhibited by OA (see Figures 2–5). The inhibitory effect of OA on the effect of saturated FAs on the p38 MAPK and ERK pathways has not been demonstrated, to our knowledge, until now. On the other hand, the inhibitory effect of OA on palmitate-induced ER stress was already documented in BRIN-BD11 and INS-1E rat pancreatic β-cells [7,28]. However, our study concerning the effect of SA presented more data.

We also provided a pilot experiment (using confocal microscopy), in which we tested the effect of OA on the SA-induced activation of the ATF6 pathway of ER stress signaling. We found (data not shown) that SA-induced ATF6 activation and translocation into the nucleus did not seem to be inhibited by OA co-incubation. Separate administration of OA had no effect on ATF6 translocation. However, this finding was not confirmed by an independent method.

Our findings concerning both MAP kinase signaling pathways as well as ER stress signaling pathways support the fact that the point of OA inhibitory intervention in SA-induced apoptotic signaling is located upstream of this signaling. Our preliminary data, as well as some previous studies [29,30], indicate that the point of intervention may be located on the plasma membrane where FAs affect membrane fluidity [31]. We hypothesize that saturated FAs with rigid and straight acyl chain conformation reduces membrane fluidity after incorporation into the lipid bilayer. This could alter the capability of membrane receptor(s) (receptor tyrosine kinases) to dimerize and thus transfer signals. Signals in the case of receptor tyrosine kinases can be signals for the

stimulation of proliferation and/or pro-survival signals. Concerning a possible mechanism of OA inhibitory intervention in SA signaling leading to apoptosis induction, we speculate that the inhibitory effect of OA can be generated simply by increasing membrane fluidity, which compensate for the effect of SA.

In conclusion, we have shown, employing human pancreatic β-cells NES2Y, that unsaturated OA is able to inhibit the effects of saturated SA on the activation of the p38 MAPK signaling pathway and inhibition of the ERK signaling pathway, as well as activation of the IRE1α and PERK ER stress signaling pathways. OA alone had minimal or no effects on tested signaling in NES2Y cells. The point of OA inhibitory intervention in SA-induced apoptotic signaling thus seems to be located upstream of the discussed signaling pathways.

4. Materials and Methods

4.1. Materials

Chemicals were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. The following primary and secondary antibodies for Western blot analysis were used: anti-phospho-MKK3/6 (#9236), anti-p38 MAPK (#8690), anti-phospho-p38 MAPK (#4511), anti-phospho-MAPKAPK-2 (#3007), anti-phospho-c-Raf (#9427), anti-phospho-MEK1/2 (#9154), anti-ERK1/2 (#5013), anti-phospho-ERK1/2 (#4370), anti-BiP (#3177), anti-CHOP (#2895), anti-eIF2α (#9722), anti-phospho-eIF2α (#9261), anti-IRE1α (#3294), anti-SAPK/JNK (#9258), anti-phospho-SAPK/JNK (#4668), anti-c-Jun (#9165) and anti-phospho-c-Jun (#9261), anti-PARP (#9542), anti-cleaved caspase-7 (#9491), anti-cleaved caspase-8 (#9496), anti-cleaved caspase-9 (#9505) from Cell Signaling Technology (Danvers, MA, USA), anti-phospho-IRE1α (ab 48187) and anti-GAPDH (ab9485) from Abcam (Cambridge, UK), and anti-actin (clone AC-40).

4.2. Cells and Culture Conditions

The human pancreatic β-cell line NES2Y employed in the experiments was kindly provided by Roger F. James (Department of Infection, Immunity and Inflammation, University of Leicester) [5,32]. NES2Y cells are proliferating cells secreting insulin with a defect in glucose responsiveness. Cells were regularly maintained in an RPMI 1640-based culture medium [33]. A defined serum-free medium [34] supplemented with FAs (1 mM SA, a combination of 1 mM SA and 0.2 mM OA, or 0.2 mM OA alone) bound to a 2% FA-free bovine serum albumin (BSA) was used in experiments [5]. Stock solutions containing FAs bound to 10% BSA in a serum-free medium were made as described previously [5] and diluted to the required concentration of FA and BSA prior to experiments. Molar ratios of FA/BSA used in experiments were lower than the ratios known to exceed the binding capacity of BSA [35].

Our previous studies showed that SA, at a concentration of 1 mM, leads to the activation of the p38 MAPK and ER stress signaling pathways and to the inhibition of the ERK signaling pathway within 24 h of the treatment [8,14]. Therefore, all assessments were performed within 24 h of treatment, except for the assessment of cell growth and viability. Since the physiological concentration of SA in adult serum seems to vary between 0.110–1.170 mM [36,37], we used 1 mM concentration of SA to simulate an elevated level of SA. An increased level of circulating FAs is a common characteristic of obese individuals [38], and a connection between obesity and type 2 diabetes mellitus (in addition to insulin resistance) has been clearly presented [38,39]. Finally, 0.2 mM concentration of OA was used, since this was the lowest concentration sufficient to inhibit the detrimental effects of SA [8].

4.3. Assessment of the Effect of Oleic Acid on the Effects of Stearic Acid on Cell Growth and Viability

Cells were seeded into the wells of a 96-well plate at a concentration of 5×10^3 cells/100 μL of culture media. After a 24-h pre-incubation period (allowing cells to attach) the culture medium was substituted with a serum-free medium containing 2% BSA with or without fatty acid(s). The control medium contained 2% BSA only. After 96 h of incubation, the number of living cells was assessed using a hemocytometer counting system, after staining with trypan blue.

4.4. Western Blot Analysis

Cells (approximately 1 × 10^6 cells per sample) were seeded and, after a 24-h pre-incubation period (allowing cells to attach), the culture medium was substituted with a serum-free medium containing 2% BSA with or without FA(s) (SA, a combination of SA and OA, or OA alone) at required concentrations. The control medium contained 2% BSA only. After the required incubation period, cells were harvested and Western blot analysis was performed as described previously [8]. All primary antibodies were used in a 1:1000 dilution. The chemiluminescent signal was detected using a Carestream Gel Logic 4000 PRO Imaging System equipped with Carestream Molecular Imaging Software (Carestream Health, New Haven, CT, USA), which was used for image acquisition. Image Master™ 2D Platinum 6.0 software (GE Healthcare, Uppsala, Sweden) was used to obtain data for densitometric analyses.

4.5. Assessment of XBP1 mRNA Splicing

Cells (approximately 1 × 10^6 cells per sample) were seeded and, after 24 h pre-incubation, FAs and thapsigargin were applied as described above (see Section 4.4). After 3, 6, 12, and 24 h incubation, the cells were harvested and the splicing of XBP1 mRNA was ascertained by RT-PCR as described previously [8,40] using GAPDH as a housekeeping gene [41].

4.6. Statistical Analysis

The statistical significance of observed differences was determined using the Tukey test.

Acknowledgments: This work was supported by research projects GAUK 1270213, PRVOUK P31, PROGRES Q36 and UNCE 204015 from the Charles University, Prague, Czech Republic, and by grant 14-00630P from the Grant Agency of the Czech Republic. We thank Roger F. James (Department of Infection, Immunity, and Inflammation, University of Leicester) for providing the NES2Y cell line and for reading the manuscript.

Author Contributions: Jan Šrámek carried out Western blot experiments and wrote the manuscript; Vlasta Němcová-Fürstová helped with Western blot experiments; Nela Pavlíková carried out densitometric analyses; Jan Kovář coordinated experiments and helped to complete the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ATF6	Activating transcription factor 6
Bcl-2	B-cell lymphoma
BiP	Immunoglobulin heavy chain-binding protein
CHOP	CCAAT-enhancer-binding protein (C/EBP) homologous protein
eIF2α	Eukaryotic initiation factor 2α
ERK1/2	Extracellular signal-regulated kinase 1/2
ER	Endoplasmic reticulum
FA	Fatty acid
Foxo03a	Forkhead box 03a
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
IRE1α	Inositol-requiring protein 1
JNK	c-Jun N-terminal kinase
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MAPKAPK	MAP kinase-activated protein kinase
MEK1/2	Mitogen-activated protein kinase/ERK kinase
NF-κB	Nuclear factor kappa B
PARP	Poly ADP-ribose polymerase
PERK	Protein kinase RNA (PKR)-like ER kinase
SA	Stearic acid
SEM	Standard error of the mean
SOS	Son of sevenless

TG	Thapsigargin
T2DM	Type 2 diabetes mellitus
OA	Oleic acid
XBP1	X-box binding protein 1

References

1. Lupi, R.; Dotta, F.; Marselli, L.; Del Guerra, S.; Masini, M.; Santangelo, C.; Patane, G.; Boggi, U.; Piro, S.; Anello, M.; et al. Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes* **2002**, *51*, 1437–1442.
2. Maedler, K.; Oberholzer, J.; Bucher, P.; Spinas, G.A.; Donath, M.Y. Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. *Diabetes* **2003**, *52*, 726–733.
3. Azevedo-Martins, A.K.; Monteiro, A.P.; Lima, C.L.; Lenzen, S.; Curi, R. Fatty acid-induced toxicity and neutral lipid accumulation in insulin-producing RINm5F cells. *Toxicol. In Vitro* **2006**, *20*, 1106–1113.
4. Welters, H.J.; Diakogiannaki, E.; Mordue, J.M.; Tadayyon, M.; Smith, S.A.; Morgan, N.G. Differential protective effects of palmitoleic acid and cAMP on caspase activation and cell viability in pancreatic beta-cells exposed to palmitate. *Apoptosis* **2006**, *11*, 1231–1238.
5. Fürstova, V.; Kopska, T.; James, R.F.; Kovar, J. Comparison of the effect of individual saturated and unsaturated fatty acids on cell growth and death induction in the human pancreatic β-cell line NES2Y. *Life Sci*. **2008**, *82*, 684–691.
6. Welters, H.J.; Tadayyon, M.; Scarpello, J.H.; Smith, S.A.; Morgan, N.G. Mono-unsaturated fatty acids protect against beta-cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure. *FEBS Lett*. **2004**, *560*, 103–108.
7. Diakogiannaki, E.; Welters, H.J.; Morgan, N.G. Differential regulation of the endoplasmic reticulum stress response in pancreatic beta-cells exposed to long-chain saturated and monounsaturated fatty acids. *J. Endocrinol.* **2008**, *197*, 553–563.
8. Nemcova-Fürstova, V.; James, R.F.L.; Kovar, J. Inhibitory effect of unsaturated fatty acids on saturated fatty acids-induced apoptosis in human pancreatic β-cells: Activation of caspases and ER stress induction. *Cell. Physiol. Biochem*. **2011**, *27*, 525–538.
9. Biden, T.J.; Boslem, E.; Chu, K.Y.; Sue, N. Lipotoxic endoplasmic reticulum stress, β cell failure, and type 2 diabetes mellitus. *Trends Endocrinol. Metab.* **2014**, *25*, 389–398.
10. Simon, M.N.; Azevedo-Martins, A.K.; Amanso, A.M.; Carvalho, C.R.O.; Curim, R. Persistent activation of Akt or ERK prevents the toxicity induced by saturated and polyunsaturated fatty acids in RINm5F beta-cells. *Toxicol. In Vitro* **2008**, *22*, 1018–1024.
11. Cvjeticanin, T.; Stojanovic, I.; Timotijevic, G.; Stosic-Grujicic, S.; Miljkovic, D. T cells cooperate with palmitic acid in induction of beta cell apoptosis. *BMC Immunol*. **2009**, *29*, 1–10.
12. Plaisance, V.; Perret, V.; Favrea, D.; Abderrahmania, A.; Yanga, J.-Y.; Widmanna, C.; Regazzi, R. Role of the transcriptional factor C/EBP in free fatty acid-elicited beta cell failure. *Mol. Cell. Endocrinol*. **2009**, *305*, 47–55.
13. Yuan, H.; Zhang, X.; Huang, X.; Lu, Y.; Tang, W.; Man, Y.; Wang, S.; Xi, J.; Li, J. NADPH Oxidase 2-Derived Reactive Oxygen Species Mediate FFAs-Induced Dysfunction and Apoptosis of β-Cells via JNK, p38 MAPK and p53 Pathways. *PLoS ONE* **2010**, *5*, e15726.
14. Sramek, J.; Nemcova-Fürstova, V.; Balusikova, K.; Daniel, P.; Jelinek, M.; James, R.F.; Kovar, J. p38 MAPK is activated but does not play a key role during apoptosis induction by saturated fatty Acid in human pancreatic β-cells. *Int. J. Mol. Sci*. **2016**, *17*, 159.
15. Cnop, M.; Ladrière, L.; Igoillo-Esteve, M.; Moura, R.F.; Cunha, D.A. Causes and cures for endoplasmic reticulum stress in lipotoxic β-cell dysfunction. *Diabetes Obes. Metab.* **2010**, *12*, S76–S82.
16. Fonseca, S.G.; Gromada, J.; Urano, F. Endoplasmic reticulum stress and pancreatic β-cell death. *Trends Endocrinol. Metab.* **2011**, *22*, 266–274.
17. Zarubin, T.; Han, J. Activation and signaling of the p38 MAP kinase pathway. *Cell Res.* **2005**, *15*, 11–18.
18. Ben-Levy, R.; Hooper, S.; Wilson, R.; Paterson, H.F.; Marshall, C.J. Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2. *Curr. Biol*. **1998**, *8*, 1049–1057.

19. Sakai, N.; Wada, T.; Furuichi, K.; Iwata, Y.; Yoshimoto, K.; Kitagawa, K.; Kokubo, S.; Kobayashi, M.; Takeda, S.; Kida, H.; et al. p38 MAPK phosphoryloation and NF-κB activation in human crescentic glomerulonephris. *Nephrol. Dial. Transplant*. **2002**, *17*, 998–1004.
20. Zhuang, S.; Demirs, J.T.; Kochevar, I.E. p38 mitogen-activated protein kinase mediates bid cleavage, mitochondrial dysfunction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. *J. Biol. Chem*. **2000**, *275*, 25939–25948.
21. Chang, F.; Steelman, L.S.; Lee, J.T.; Shelton, J.G.; Navolanic, P.M.; Blalock, W.L.; Franklin, R.A. McCubrey, J.A. Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: Potential targeting for therapeutic intervention. *Leukemia* **2003**, *17*, 1263–1293.
22. Lu, Z.; Xu, S. ERK1/2 MAP kinases in cell survival and apoptosis. *IUBMB Life* **2006**, *58*, 621–631.
23. Hetz, C.; Martinon, F.; Rodriguez, D.; Glimcher, L.H. The unfolded protein response: Integrating stress signals through the stress sensor IRE1α. *Physiol. Rev.* **2011**, *91*, 1219–1243.
24. Tabas, I.; Ron, D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat. Cell. Biol.* **2011**, *13*, 184–190.
25. Nemcova-Fürstova, V.; Balusikova, K.; Sramek, J.; James, R.F.; Kovar, J. Caspase-2 and JNK Activated by Saturated Fatty Acids are Not Involved in Apoptosis Induction but Modulate ER Stress in Human Pancreatic β-cells. *Cell. Physiol. Biochem*. **2013**, *31*, 277–289.
26. Newsholme, P.; Keane, D.; Welters, H.J.; Morgan, N.G. Life and death decisions of the pancreatic beta-cell: The role of fatty acids. *Clin. Sci.* **2007**, *112*, 27–42.
27. Morgan, N.G. Fatty acids and beta-cell toxicity. *Curr. Opin. Clin. Nutr. Metab. Care* **2009**, *12*, 117–122.
28. Sommerweiss, D.; Gorski, T.; Richter, S.; Garten, A.; Kiess, W. Oleate rescues INS-1E β-cells from palmitate-induced apoptosis by preventing activation of the unfolded protein response. *Biochem. Biophys. Res. Commun.* **2013**, *441*, 770–776.
29. Vacaresse, N.; Lajoie-Mazenc, I.; Auge, N.; Suc, I.; Frisach, M.-F.; Salvayre, R.; Nègre-Salvayre, A. Activation of epithelial growth factor receptor pathway by unsaturated fatty acids. *Circ. Res.* **1995**, *85*, 892–899.
30. Holzer, R.G.; Park, E.J.; Li, N.; Tran, H.; Chen, M.; Choi, C.; Solinas, G.; Karin, M. Saturated fatty acids induce c-Src clustering within membrane subdomains, leading to JNK activation. *Cell* **2011**, *147*, 173–184.
31. Karnovsky, M.J.; Kleinfeld, A.M.; Hoover, R.L.; Klausner, R.D. The concept of lipid domains in membranes. *J. Cell. Biol*. **1982**, *94*, 1–6.
32. Macfarlane, W.M.; Cragg, H.; Docherty, H.M.; Read, M.L.; James, R.F.; Aynsley-Green, A.; Docherty, K. Impaired expression of transcription factor IUF1 in a pancreatic beta-cell line derived from a patient with persistent hyperinsulinaemic hypoglycaemia of infancy (nesidioblastosis). *FEBS Lett*. **1997**, *413*, 304–308.
33. Musilkova, J.; Kovar, J. Additive stimulatory effect of extracellular calcium and potassium on non-transferrin ferric iron uptake by HeLa and K562 cells. *Biochim. Biophys. Acta* **2001**, *1514*, 117–126.
34. Kovar, J.; Franek, F. Growth-stimulating effect of transferrin on a hybridoma cell line: Relation to transferrin iron-transporting function. *Exp. Cell. Res*. **1989**, *182*, 358–369.
35. Cnop, M.; Hannaert, J.C.; Hoorens, A.; Eizirik, D.L.; Pipeleers, D.G. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* **2001**, *50*, 1771–1777.
36. Lagerstedt, S.A.; Hinrichs, D.R.; Batt, S.M.; Magera, M.J.; Rinaldo, P.; McConnell, J.P. Quantitative determination of plasma c8-c26 total fatty acids for the biochemical diagnosis of nutritional and metabolic disorders. *Mol. Genet. Metab.* **2001**, *73*, 38–45.
37. Abdelmagid, S.A.; Clarke, S.E.; Nielsen, D.E.; Badawi, A.; El-Sohemy, A.; Mutch, D.M.; Ma, D.W.L. Comprehensive profiling of plasma fatty acid concentrations in young healthy Canadian adults. *PLoS ONE* **2015**, *10*, e0116195.
38. Kahn, S.E.; Hull, R.L.; Utzschneider, K.M. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* **2006**, *444*, 840–846.
39. Steyn, N.P.; Mann, J.; Bennett, P.H.; Temple, N.; Zimmet, P.; Tuomilehto, J.; Lindstrom, J.; Louheranta, A. Diet, nutrition and the prevention of type 2 diabetes. *Public Health Nutr*. **2004**, *7*, 147–165.

40. Gao, Y.Y.; Liu, B.Q.; Niu, X.F.; Zhuang, Y.; Wang, H.Q. Resveratrol-induced cytotoxicity in human Burkitt's lymphoma cells is coupled to the unfolded protein response. *BMC Cancer* **2010**, *10*, 445.
41. Balusikova, K.; Neubauerova, J.; Dostalikova-Cimburova, M.; Horak, J.; Kovar, J. Differing expression of genes involved in non-transferrin iron transport across plasma membrane in various cell types under iron deficiency and excess. *Mol. Cell. Biochem.* **2009**, *321*, 123–133.

7.5. PUBLIKACE 5

Šrámek J, Polák J, Němcová-Fürstová V, Kovář J:
Hypoxia modulates effects of fatty acids on human pancreatic β-cells
International Journal of Molecular Sciences, připraveno k odeslání, 2018.

Communication

Hypoxia modulates effects of fatty acids on human pancreatic β-cells

Jan Šrámek [1], Jan Polák [2], Vlasta Němcová-Fürstová [1] and Jan Kovář [1,*]

[1] Division of Cell and Molecular Biology & Center for Research of Diabetes, Metabolism and Nutrition, Third Faculty of Medicine, Charles University, Ruská 87, Prague, 110 00, Czech Republic; jan.sramek@lf3.cuni.cz (J.Š.); vlasta.furstova@centrum.cz (V.N.-F.); jan.kovar@lf3.cuni.cz (J.K.)

[2] Department for the Study of Obesity and Diabetes, Third Faculty of Medicine, Charles University, Ruska 87, CZ-100 00 Prague, Czech Republic; jan.polak@lf3.cuni.cz (J.P.)

* Correspondence: jan.kovar@lf3.cuni.cz (J.K.); Tel.: +420-267-102-658 (J.K.)

Academic Editor: name
Received: date; Accepted: date; Published: date

Abstract: Saturated fatty acids (FAs) induce apoptosis in the human pancreatic β-cell line NES2Y while unsaturated FAs have nearly no detrimental effect. Moreover, unsaturated FAs are capable of inhibiting the pro-apoptotic effect of saturated FAs. Hypoxia is also known to have deleterious effects on β-cells function and viability. In the present study, we tested modulatory effect of hypoxia on the effect of FAs on growth and viability of human pancreatic β-cells NES2Y. We showed that hypoxia increased pro-apoptotic effect of stearic acid (SA). Endoplasmic reticulum stress signaling seemed to be involved in this effect. Hypoxia also decreased protective effect of oleic acid (OA) against pro-apoptotic effect of SA. Thus OA in the presence of hypoxia was unable to safe SA-treated β-cells from apoptosis induction. Hypoxia itself had only weak detrimental effects on NES2Y cells. Our data suggest that hypoxia could represent an important factor in pancreatic β-cell death induced by FAs and thus in the development of type 2 diabetes mellitus.

Keywords: fatty acids; pancreatic β-cells; hypoxia; apoptosis; ER stress; caspases; NES2Y.

1. Introduction

It was suggested that increased levels of fatty acids (FAs) in blood as well as hypoxia are factors contributing to pancreatic β-cells failure and death and thus to type 2 diabetes mellitus (T2DM) development [Lupi et al. 2002, Maedler et al. 2003, Azevedo-Martins et al. 2006, Welters 2006, Fürstová et al. 2008, Lai et al. 2009; Zheng et al. 2012; Fang et al. 2014].

Our previous studies as well as studies of others have shown that the effect of FAs on the function and viability of pancreatic β-cells depends on the level of their saturation. Saturated FAs, i.e. palmitic or more efficiently stearic acid (SA), have detrimental effect on β-cells, whereas unsaturated FAs, i.e. oleic acid (OA) or palmitoleic acid, are well tolerated and are even capable of inhibiting the pro-apoptotic effect of saturated FAs [Maedler et al. 2003, Welters et al. 2004, 2006, Fürstová et al. 2008, Diakogiannaki et al. 2008, Němcová-Fürstová et al. 2011].

Direct hypoxia detrimental effects on pancreatic β-cell function and viability was documented in rodents [Lai et al. 2009; Zheng et al. 2012; Fang et al. 2014]. The role of hypoxia in β-cell apoptosis can be supported by the presence of hypoxia *in vivo* in the islets of animal models of T2DM [Jonas et al. 1999; Li et al. 2006; Sato et al. 2011; Bensellam et al. 2012b]. In addition, it was found that severe hypoxia in islet grafts contributes to β-cell apoptosis in the early post-transplantation period [Emamaulee et al. 2007; Olsson et al. 2011].

Molecular mechanisms of apoptosis induction by saturated FAs and inhibition of this induction by unsaturated FAs as well as molecular mechanisms underlying the deleterious effects

of hypoxia in β-cells remain unclear [Bensellam et al. 2012a, Biden et al. 2014]. However, beside caspases activation leading to PARP cleavage endoplasmic reticulum (ER) stress signaling seems to be involved [Cnop et al. 2010, Němcová-Fürstová et al. 2011, Zheng et al. 2012; Bensellam et al. 2016].

ER stress was demonstrated to result in activation of three signaling pathways. These pathways primarily participate in the restoration of ER homeostasis, e.g. by increasing the expression of chaperones, such as immunoglobulin heavy chain-binding protein (BiP) [Hetz et al. 2012]. However, if this response fails, caspases are activated and apoptosis is induced by mechanisms that are not still completely understood. Suggested mediator is here transcription factor CCAAT-enhancer-binding protein (C/EBP) homologous protein (CHOP) [Tabas & Ron 2011]. Proteins BiP and CHOP are therefore considered to be main markers of ER stress (reviewed in [Biden et al. 2014]).

In the present study, we tested modulatory effect of hypoxia on the effect of FAs on growth and viability of human pancreatic β-cells NES2Y. This seems to be the first study testing such hypoxia effect in pancreatic β-cells as well as in other cell types. We documented that hypoxia increased pro-apoptotic effect of SA. ER stress signaling seemed to be involved in this effect. Hypoxia also decreased protective effect of OA against pro-apoptotic effect of SA. Thus OA in the presence of hypoxia was unable to safe SA-treated β-cells from apoptosis induction. Hypoxia itself had only weak detrimental effects on NES2Y cells. These results suggest that hypoxia could represent an important factor in pancreatic β-cell death induced by FAs and thus in the development of T2DM.

2. Results

2.1. Modulation of the effect of fatty acids on cell growth and viability by hypoxia

In non-treated cells only strong hypoxia decreased the number of living cells approximately 1.5-fold comparing to the number of cells under normoxia within 48 h of incubation (Figure 1A).

Under control conditions (20 % O_2), 1 mM stearic acid (SA) decreased the number of living NES2Y cells approximately to 28 % of the number of non-treated cells, i.e. significantly below the number of cells of inoculum, within 48 h of incubation. Moderate hypoxia (4 % O_2) produced further significant decrease of the number of cells treated with 1 mM SA within the same incubation period. The number of living cells under moderated hypoxia was about 10 % of the number of cells under normoxia. Ratio of the number of SA-treated cells and non-treated cells was decreased from 0.082 (normoxia) to 0.029 by moderate hypoxia. Strong hypoxia (1 % O_2) decreased the number of cells treated with SA more than moderate hypoxia. The number of living cells under strong hypoxia represented approximately 4 % of the number of cells under normoxia. Ratio of the number of SA-treated cells and non-treated cells was decreased from 0.082 (normoxia) to 0.017 by strong hypoxia (see Figure 1A, B).

Under control conditions (20 % O_2), 0.2 mM oleic acid (OA) applied together with 1 mM SA increased the number of living cells approximately 6.5-fold compared to the number of cells treated with SA only, i.e. significantly over the number of cells of inoculum, within 48 h of incubation. Moderate hypoxia significantly decreased this enhancing effect of OA. The number of living cells under moderated hypoxia was increased due to OA co-application with SA only 5.4-fold comparing to the number of cells treated with SA only, i.e. significantly below the number of cells of inoculum. Strong hypoxia decreased the enhancing effect of OA more than moderate hypoxia. The number of living cells under strong hypoxia was increased after OA co-application only 3.1-fold comparing to the number of cells treated with SA only (see Figure 1A, C).

OA at a concentration of 0.2 mM had no effect on the number of living cells under control conditions (20 % O_2) within 48 h of incubation. Hypoxia seemed to have similar effect on OA-treated cells like on non-treated cells (Figure 1A).

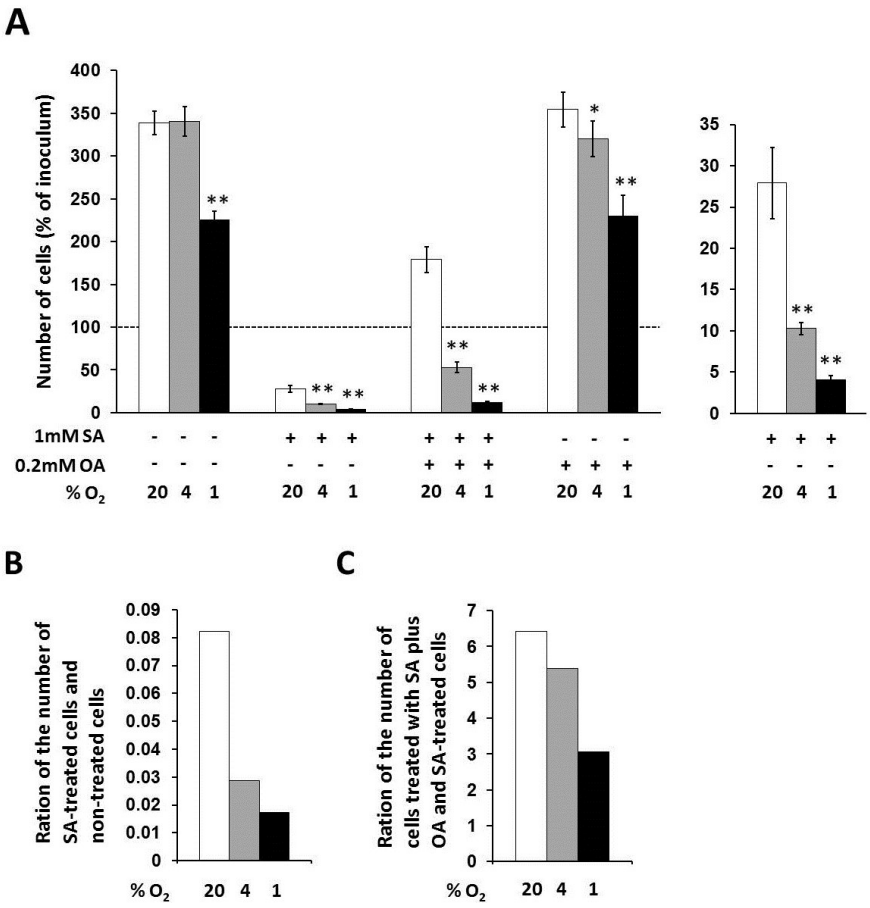


Figure 1. (**A**) Effects of hypoxia (see "Materials and Methods") applied simultaneously with 1 mM stearic acid (SA), 1 mM SA plus 0.2 mM oleic acid (OA), and 0.2 mM OA (see "Materials and Methods") on cell growth and viability of NES2Y cells. (**B**) Effect of hypoxia on cell growth and viability of NES2Y cells treated with SA when comparing with cells without fatty acid (ratio of the number of SA-treated cells and non-treated cells). (**C**) Effect of hypoxia on cell growth and viability of NES2Y cells treated with SA plus OA when comparing with SA-treated cells (ration of the number of cells treated with SA plus OA and SA-treated cells). When assessing cell growth and viability, cells were seeded at a concentration of 9×10^4 cells/100 μL of culture medium per well of 24-well plate (see "Materials and Methods"). The number of living cells was determined after 48 h of incubation in the presence of a hypoxia (4 % and 1 % O_2) or under control conditions (20 % O_2). Each column represents the mean of four separate cultures ± SEM. * $P<0.05$, ** $P<0.001$ when comparing the effect of particular hypoxia with normoxia. Dotted line represents the number of cells of inoculum.

2.2. Modulation of the effect of fatty acids on activation of caspases by hypoxia

Under control conditions (20 % O_2), the application of 1 mM SA resulted in significant activation (cleavage) of initiator caspase-8, -9 as well as executioner caspase -6, -7 and the cleavage of caspase substrate PARP in NES2Y cells compared to non-treated cells after 18 h of incubation. Moderate hypoxia (4 % O_2) seemed to decrease slightly the level of cleaved caspases but not of the PARP in cells treated with SA. Strong hypoxia (1 % O_2) increased the level of caspases and PARP cleavage (Figure 2).

Under control conditions, the application of 0.2 mM OA together with 1 mM SA decreased the cleavage of caspases and PARP due to SA application within 18 h of incubation. Moderate as well

as strong hypoxia counteracted the protection effect of OA against cleavage of caspases and PARP due to SA application (Figure 2).

The application of OA at a concentration of 0.2 mM did not result in caspase activation and PARP cleavage within 18 h of incubation. Hypoxia did not affect it. Hypoxia itself had nearly no effect on caspase activation and PARP cleavage (Figure 2).

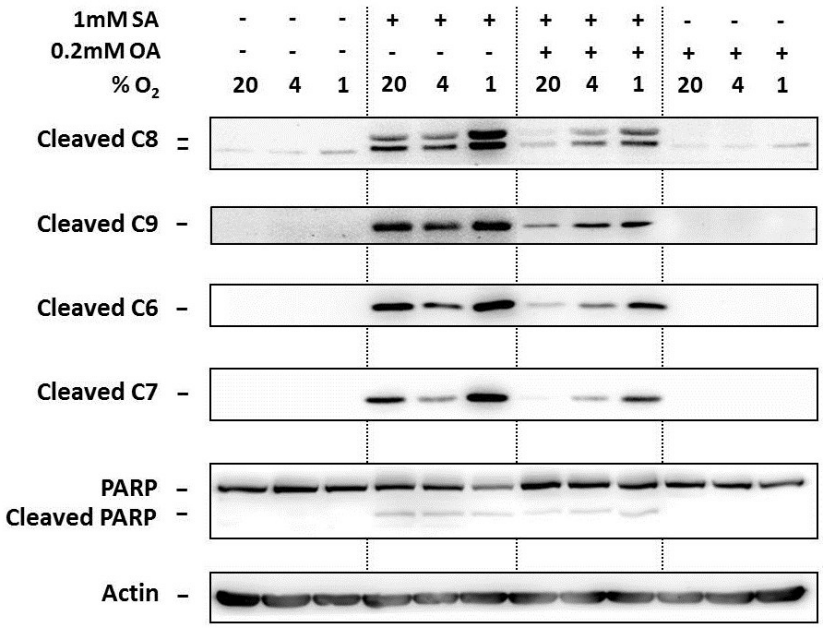


Figure 2. Effects of hypoxia (see "Materials and Methods") applied simultaneously with 1 mM stearic acid (SA), 1 mM SA plus 0.2 mM oleic acid (OA), and 0.2 mM OA (see "Materials and Methods") on the activation of caspase-8 (C8), caspase-9 (C9), caspase-6 (C6), caspase-7 (C7) assessed by the level of cleaved caspases and on the level of PARP cleavage in NES2Y cells. After 18 h of incubation, levels of individual proteins were determined using western blot analysis employing relevant antibodies (see "Materials and Methods"). Actin level was used to confirm equal protein loading. The data shown were obtained in one representative experiment from at least three independent experiments.

2.3. Modulation of the effect of fatty acids on the expression of BiP and CHOP by hypoxia

Under control conditions (20 % O_2), the application of 1 mM SA resulted in a significant increase of the expression of endoplasmic reticulum stress markers BiP and CHOP in NES2Y cells after 18 h of incubation. Moderate (4 % O_2) as well as strong (1 % O_2) hypoxia seemed to increase the expression of both proteins in the cells treated with SA (Figure 3).

Under control conditions, the application of 0.2 mM OA together with 1 mM SA decreased significantly the expression of BiP and CHOP due to SA application after 18 h of incubation. Moderate as well as strong hypoxia counteracted the effect of OA and the expression of BiP and CHOP was increased when comparing with OA effect under control conditions (Figure 3).

Under control conditions, the application of 0.2 mM OA alone had no effect on the expression of BiP as well as CHOP. Hypoxia did not change it significantly. Hypoxia itself had nearly no effect on the expression of both proteins (Figure 3).

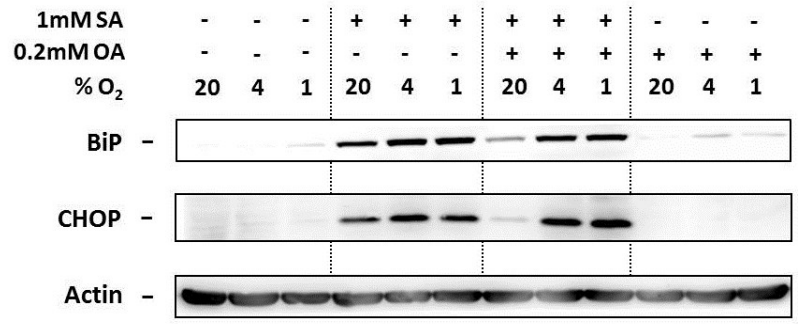


Figure 3. Effects of hypoxia (see "Materials and Methods") applied simultaneously with 1 mM stearic acid (SA), 1 mM SA plus 0.2 mM oleic acid (OA), and 0.2 mM OA (see "Materials and Methods") on the level of BiP and CHOP in NES2Y cells. After 18 h of incubation, levels of individual proteins were determined using western blot analysis employing relevant antibodies (see "Materials and Methods"). Actin level was used to confirm equal protein loading. The data shown were obtained in one representative experiment from at least three independent experiments.

3. Discussion

Previous studies have demonstrated that saturated fatty acids (FAs), e.g. stearic acid (SA) and palmitic acid, induced apoptosis in the human pancreatic β-cell line NES2Y while unsaturated FAs (oleic acid (OA) and palmitoleic acids) have weak detrimental effect only at high concentrations. Moreover, unsaturated FAs are capable of inhibiting the pro-apoptotic effect of saturated FAs [Maedler et al. 2003, Welters et al. 2004, 2006, Diakogiannaki et al. 2008, Fürstová et al. 2008, Němcová-Fürstová et al. 2011]. Beside saturated FAs also hypoxia was demonstrated to have deleterious effect on β-cells function and viability [Jonas et al. 1999, Li et al. 2006, Sato et al. 2011, Bensellam et al. 2012b, Zheng et al. 2012, Fang et al. 2014, Bensellam et al. 2016].

We tested in this study the modulation of effects of FAs on the growth, viability and functioning of the human pancreatic β-cells NES2Y by hypoxia. To our knowledge, this is the first study testing such hypoxia effect in pancreatic β-cells as well as in other cell types.

We have documented that moderate (4 % O_2) as well as strong (1 % O_2) hypoxia significantly increased pro-apoptotic effect of saturated SA in NES2Y β-cells. The effect of strong hypoxia was significantly stronger then the effect of moderate hypoxia (see Figure 1A, B and 2). Concerning these data, it seems that deleterious effects of hypoxia when combined with other pro-apoptotic factor(s) may represent decisive element leading to pancreatic β-cell death. Potentiation of cytotoxic effect of various agents due to hypoxia was published in other cell types [Walford et al. 2004, Bullova et al. 2016]. Next, we have showed that hypoxia significantly decreased protective effect of unsaturated OA against pro-apoptotic effect of saturated SA. Thus OA was unable to block apoptosis induction in SA-treated β-cells. The effect of strong hypoxia was again markedly significantly stronger then the effect of moderate hypoxia (Figure 1A, C and 2). Concerning these results, it seems that hypoxia can also inhibit anti-apoptotic effect of some agens in pancreatic β-cells. However, there were no studies published considering this issue to our knowledge. Regarding our results of the effect of hypoxia applied alone, we have found that only strong hypoxia (1% O2) has significant effect on cell growth and viability of NES2Y cells, i.e. it decreased cell growth (Figure 1A). Interestingly, in rodent β-cell lines (INS-1, MIN6) moderate or strong hypoxia caused much stronger deleterious effects [Lai et al. 2009; Zheng et al. 2012; Sato et al. 2014; Qiao et al. 2015]. Additional data are needed to elucidate if relatively weak hypoxia effect in NES2Y cells is due to impaired mechanisms that mediate detrimental effects of hypoxia or it is physiological situation and human pancreatic β-cells are more tolerant to hypoxic stress than

rodent β-cells. Taken together data discussed above suggest that hypoxia could represent an important factor in pancreatic β-cell death induced by FAs and thus in the development of type 2 diabetes mellitus (T2DM).

Concerning possible molecules mediating hypoxia detrimental effects in NES2Y cells it seem that caspases (caspase-9, -8, -7 and -6) and PARP are involved since FAs-induced cleavage of these proteins was increased under hypoxic conditions (see Figure 2A). A possible involvement of caspase-8, -3 and -7 was published till now [Lai et al. 2009; Qiao et al. 2015; Sato et al. 2014, Zheng et al. 2012, Wang et al. 2012, Bensellam et al. 2016, Ryu et al. 2009. Tian et al. 2013] but this is the first study suggesting possible participation of caspase-9 and -6 as a mediators of hypoxia deleterious effects in pancreatic β-cells. Interestingly, moderate hypoxia decreased saturated SA-induced caspases cleavage (Figure 2) and it was not followed by increase of the number of living cells. On the contrary, decrease of the number of living cells was observed (see Figure 1A). Additional studies are needed to elucidate these data. We have also documented that hypoxia increases FAs-induced expression of the main ER stress markers BiP and CHOP (Figure 3). These results as well as results of other authors [Oslowski & Urano 2011, Zheng et al. 2012] suggest that beside caspases activation also ER stress signalling mediates detrimental effects of hypoxia in pancreatic β-cells. ER stress signaling was generally considered to be upstream from caspases activation [Tabas & Ron 2011]. Therefore, hypoxia intervention into molecular mechanisms of apoptosis induction by saturated FAs and into mechanisms of inhibition of this induction by unsaturated FAs must be located at least partly within or upstream of ER stress pathways.

FA-induced ER stress is potentiated by hypoxia in β-cell probably since the appropriate folding of proinsulin in the ER involves the formation of three disulphide bonds, a process that requires molecular oxygen [Appenzeller-Herzog & Ellgaard 2008]. Therefore, lack of molecular oxygen leads to accumulation of unfolded proinsulin and other proteins decreasing β-cell function and together with FAs contributing to disruption of ER homeostasis. It was documented in cardial myocytes that strong hypoxia induces redistribution of two FAs transporters FAT/CD36 and FABPpm from an intracellular pool to plasma membranes leading to increased FAs accumulation [Chabowski et al. 2006]. Both transporters were documented to be present in pancreatic β-cells [Hyder et al. 2010, Dalgaard et al. 2011]. One can speculate that this redistribution leading to FAs uptake can occur also in β-cells in response to hypoxia treatment and thus this effect may also represent the mechanism by which hypoxia increases FAs-induced ER stress in pancreatic β-cells. Moreover, increased uptake of FAs in cells under hypoxia conditions may lead to situation when OA (at low concentrations protective, at high concentrations pro-apoptotic [Šrámek et al. 2016b]) has no more cytoprotective effects in β-cell but on the contrary has pro-apoptotic effects. And/or, OA is no more able to inhibit pro-apoptotic effects of SA whose concentrations were increased in a cell due to hypoxia-induced uptake.

To conclude, we demonstrated that hypoxia increased pro-apoptotic effect of SA in human pancreatic β-cells NES2Y. ER stress signaling could be involved here. Hypoxia decreased protective effect of OA against pro-apoptotic effect of SA. Thus in the presence of hypoxia OA was unable to safe SA-treated β-cells from apoptosis induction. Hypoxia itself had only weak detrimental effects on NES2Y cells. Our data suggest that hypoxia could represent an important factor in pancreatic β-cell death induced by FAs and thus in the development of T2DM.

4. Materials and Methods

4.1. Materials

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. For western blot analysis, the following primary and secondary antibodies were used: anti-cleaved caspase-6 (#9761), anti-cleaved caspase-7 (#9491), anti-cleaved caspase-8 (#9496), anti-cleaved caspase-9 (#9505) anti-PARP (#9542), anti-BiP (#3177), anti-CHOP (#2895) from Cell Signaling Technology (Danvers, MA, USA) and anti-actin (clone AC-40).

4.2. Cells and culture conditions

The human pancreatic β-cell line NES2Y [Macfarlane et al. 1997, Fürstová et al. 2008] was used. Cells were routinely maintained in an RPMI 1640 based culture medium [Musílková & Kovář 2001]. Cells were plated on 50 mm gas permeable culture dishes (Sarstedt AG & Co, Nümbrecht, Germany) or on 24-well gas permeable plates (Coy Laboratory Products, Grass Lake, MI, USA) according to experimental protocols described below. In experiments, a defined serum-free medium [Kovář & Franěk 1989] supplemented with fatty acids (FAs) (1 mM stearic acid (SA), a combination of 1 mM SA and 0.2 mM oleic acid (OA), or 0.2 mM OA alone) bound to a 2% FA-free bovine serum albumin (BSA) was used [Fürstová et al. 2008]. Stock solutions containing FA(s) bound to 10% BSA in a serum-free medium were prepared as described previously [Fürstová et al. 2008] and diluted to the required concentration of FA and BSA prior to experiments. FA/BSA molar ratios used in experiments were lower than the ratios known to exceed the binding capacity of BSA [Cnop et al. 2001].

Our previous studies showed that SA, at a concentration of 1 mM, induces endoplasmic reticulum stress and apoptosis in most NES2Y cells within 24 h of application [Němcová-Fürstová et al. 2011, 2013; Šrámek et al. 2016a, 2017]. Therefore, all assessments were performed within 24 h after FAs application except for the assessment of cell growth and viability. 1 mM concentration of SA was used to simulate an elevated level of SA in blood [Lagerstedt et al. 2001, Abdelmagid et al. 2015]. 0.2 mM concentration of OA was used, since this was the lowest concentration sufficient to inhibit the detrimental effects of SA [Fürstová et al. 2008].

4.3. Assessment of the effect of hypoxia on cell growth and viability

Cells were seeded at a concentration of 9×10^4 cells/100 μL of culture media into the wells of 24-well plate. After a 24-h pre-incubation period (allowing cells to attach) the culture medium was replaced with a serum-free medium containing 2% BSA with or without FA(s) (SA, a combination of SA and OA, or OA alone) at required concentrations. Cells were placed inside a standard incubation cabinet providing 20% oxygen level or inside chambers of a specific incubation cabinet [Polák et al. 2015] in which oxygen levels were 4 % or 1 %. Standard incubation cabinet (20% oxygen level) represents normoxia of outer environment. It provides sufficient oxygen concentration for normal β-cells function. Incubation in specific incubation cabinet providing 4% oxygen concentration represents moderate hypoxia for pancreatic β-cells. Incubation in 1% oxygen concentration represents strong hypoxia. We used 20% oxygen concentration as normoxia in this paper. It is commonly used also by other authors [Lai et al. 2009, Zheng et al. 2012, Sato et al. 2014]. After 48 h of incubation, the number of living cells was determined using a hemocytometer counting system, after staining with trypan blue.

4.4. Western blot analysis

Cells (approximately 1×10^6 cells per sample) were seeded and after a 24-h pre-incubation period (allowing cells to attach) the culture medium was replaced with a serum-free medium containing 2% BSA with or without FA(s) (SA, a combination of SA and OA, or OA alone) at required concentrations. Cells were placed inside a standard incubation cabinet (20 % oxygen level, normoxia) or inside chambers of a specific incubation cabinet [Polák et al. 2015] in which oxygen levels were 4 % (moderate hypoxia) or 1 % (strong hypoxia). After the required incubation period, cells were harvested and western blot analysis was performed as described previously [Němcová-Fürstová et al. 2011]. All primary antibodies were used in a 1:1000 dilution. The chemiluminescent signal was detected using a Carestream Gel Logic 4000 PRO Imaging System equipped with Carestream Molecular Imaging Software (Carestream Health, New Haven, CT, USA), which was used for image acquisition.

4.5. Statistical analysis

The statistical significance of observed differences was determined using the Student´s t-test. $p < 0.05$ was considered statistically significant.

Acknowledgments: This work was supported by research projects PRVOUK P31, PROGRES Q36 and UNCE 204015 of the Charles University, Prague, Czech Republic, and by grant 18-10144S from the Grant Agency of the Czech Republic. We thank Roger F. James (Department of Infection, Immunity, and Inflammation, University of Leicester) for providing the NES2Y cell line.

Author Contributions: J.Š. carried out all experiments and wrote the manuscript; V.N.-F. helped with western blot experiments; J.P. and J.K. coordinated experiments and helped to complete the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

BiP	Immunoglobulin heavy chain-binding protein
CHOP	CCAAT-enhancer-binding protein (C/EBP) homologous protein
ER	Endoplasmic reticulum
FAs	Fatty acids
SA	Stearic acid
T2DM	Type 2 diabetes mellitus
OA	Oleic acid

References

1. Lupi, R.; Dotta, F.; Marselli, L.;, Del Guerra, S.; Masini, M.; Santangelo, C.; Patane, G.; Boggi, U.; Piro, S.; Anello, M.; Bergamini, E.; Mosca, F.; Di Mario, U.; Del Prato, S.; Marchetti, P. Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes* **2002**, *51,* 1437–1442.
2. Maedler, K.; Oberholzer, J.; Bucher, P.; Spinas, G.A.; Donath, M.Y. Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. *Diabetes* **2003**, *52*, 726–733.
3. Azevedo-Martins, A.K.; Monteiro, A.P.; Lima, C.L.; Lenzen, S.; Curi, R. Fatty acid-induced toxicity and neutral lipid accumulation in insulin-producing RINm5F cells. *Toxicol. In Vitro* **2006**, *20,* 1106–1113.
4. Welters, H.J.; Diakogiannaki, E.; Mordue, J.M.; Tadayyon, M.; Smith, S.A.; Morgan, N.G. Differential protective effects of palmitoleic acid and cAMP on caspase activation and cell viability in pancreatic beta-cells exposed to palmitate. *Apoptosis* **2006**, *11*, 1231–1238.
5. Fürstova, V.; Kopska, T.; James, R.F.; Kovar, J. Comparison of the effect of individual saturated and unsaturated fatty acids on cell growth and death induction in the human pancreatic β-cell line NES2Y. *Life Sci*. **2008**, *82*, 684–691.
6. Welters, H.J.; Tadayyon, M.; Scarpello, J.H.; Smith, S.A.; Morgan, N.G. Mono-unsaturated fatty acids protect against beta-cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure. *FEBS Lett*. **2004**, *560*, 103–108.
7. Diakogiannaki, E.; Welters, H.J.; Morgan, N.G. Differential regulation of the endoplasmic reticulum stress response in pancreatic beta-cells exposed to long-chain saturated and monounsaturated fatty acids. *J. Endocrinol. Jun*. **2008**, *197*, 553–563.
8. Nemcova-Fürstova, V.; James, R.F.L.; Kovar, J. Inhibitory effect of unsaturated fatty acids on saturated fatty acids-induced apoptosis in human pancreatic β-cells: activation of caspases and ER stress induction. *Cell. Physiol. Biochem*. **2011**, *27*, 525–538.
9. Biden, T.J.; Boslem, E.; Chu, K.Y.; Sue, N. Lipotoxic endoplasmic reticulum stress, β cell failure, and type 2 diabetes mellitus. *Trends Endocrinol. Metab*. **2014**, *pii*, S1043–2760.
10. Cvjeticanin, T.; Stojanovic, I.; Timotijevic, G.; Stosic-Grujicic, S.; Miljkovic, D. T cells cooperate with palmitic acid in induction of beta cell apoptosis. *BMC Immunol*. **2009**, *29*, 1–10.
11. Hetz, C.; Martinon, F.; Rodriguez, D.; Glimcher, L.H. The unfolded protein response: integrating stress signals through the stress sensor IRE1α. *Physiol. Rev.* 2011, 91, 1219–1243.
12. Macfarlane, W.M.; Cragg, H.; Docherty, H.M.; Read, M.L.; James, R.F.; Aynsley-Green, A.; Docherty, K. Impaired expression of transcription factor IUF1 in a pancreatic beta-cell line derived from a

patient with persistent hyperinsulinaemic hypoglycaemia of infancy (nesidioblastosis). *FEBS Lett*. **1997**, *413*, 304–308.

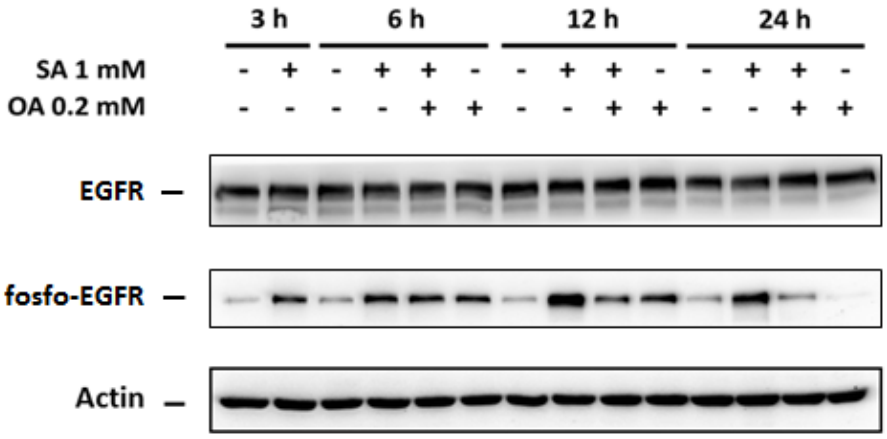
13. Musilkova, J.; Kovar, J. Additive stimulatory effect of extracellular calcium and potassium on non-transferrin ferric iron uptake by HeLa and K562 cells. *Biochim. Biophys. Acta* **2001**, *1514*, 117–126.
    Kovar, J.; Franek, F. Growth-stimulating effect of transferrin on a hybridoma cell line: relation to transferrin iron-transporting function. *Exp. Cell. Res*. **1989**, *182*, 358–369.
14. Cnop, M.; Hannaert, J.C.; Hoorens, A.; Eizirik, D.L.; Pipeleers, D.G. Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* **2001**, *50*, 1771–1777.
15. Bensellam, M.; Laybutt, D.R.; Jonas, J.C. The molecular mechanisms of pancreatic β-cell glucotoxicity: recent findings and future research directions. *Mol. Cell. Endocrinol*. **2012b**, 364, 1–27.
16. Valencia-Flores, M.; Orea, A.; Castano, V.A.; Resendiz, M.; Rosales, M.; Rebollar, V.; Santiago, V.; Gallegos, J.; Campos, R.M.; Gonzalez, J.; Oseguera, J.; Garcia-Ramos, G.; Bliwise, D.L. Prevalence of sleep apnea and electrocardiographic disturbances in morbidly obese patients. *Obes. Res*. **2000**, 8, 262–269.
17. Olsson, R.; Olerud, J.; Pettersson, U.; Carlsson, P.O. Increased numbers of low-oxygenated pancreatic islets after intraportal islet transplantation. *Diabetes* **2011**, 60, 2350–2353.
18. Bensellam, M.; Maxwell, E.L.; Chan, J.Y.; Luzuriaga, J.; West, P.K.; Jonas, J.C.; Gunton, J.E.; Laybutt, D.R. Hypoxia reduces ER-to-Golgi protein trafficking and increases cell death by inhibiting the adaptive unfolded protein response in mouse beta cells. *Diabetologia* **2016**, 59, 1492–1502.
19. Fang, Y.; Zhang, Q.; Tan, J.; Li, L.; An, X.; Lei, P. Intermittent hypoxia-induced rat pancreatic β-cell apoptosis and protective effects of antioxidant intervention. *Nutr. Diabetes* **2014**, 4, e131.
20. Jonas, J.C.; Sharma, A.; Hasenkamp, W.; Ilkova, H.; Patanè, G.; Laybutt, R.; Bonner-Weir, S.; Weir, G.C. Chronic hyperglycemia triggers loss of pancreatic b cell differentiation in an animal model of diabetes. *J. Biol. Chem*. **1999**, 274, 14112–14121.
21. Li, X.; Zhang, L.; Meshinchi, S.; Dias-Leme, C.; Raffin, D.; Johnson, J.D.; Treutelaar, M.K.; Burant, C.F. Islet microvasculature in islet hyperplasia and failure in a model of type 2 diabetes. *Diabetes* **2006**, 55, 2965–2973.
22. Sato, Y.; Endo, H.; Okuyama, H. Takeda, T.; Iwahashi, H.; Imagawa, A.; Yamagata, K.; Shimomura, I.; Inoue, M. Cellular hypoxia of pancreatic b-cells due to high levels of oxygen consumption for insulin secretion in vitro. *J. Biol. Chem*. **2011**, 286, 12524–12532.
23. Bensellam, M.; Duvillie, B.; Rybachuk, G.; Laybutt, D.R.; Magnan, C.; Guiot, Y.; Pouysségur, J.; Jonas, J.C. Glucoseinduced O2 consumption activates hypoxia inducible factors 1 and 2 in rat insulin-secreting pancreatic beta-cells. *PLoS One* **2012a**, 7, e29807.
24. Zheng, X.; Zheng, X.; Wang, X.; Ma, Z.; Gupta Sunkari, V.; Botusan, I.; Takeda, T.; Björklund, A.; Inoue, M.; Catrina, S.B.; Brismar, K.; Poellinger, L.; Pereira, T.S. Strong hypoxia induces apoptosis of pancreatic beta-cell by activation of the unfolded protein response and upregulation of CHOP. *Cell Death Dis*. **2012**, 3, e322.
25. Emamaullee, J.A.; Shapiro, A.M. Factors influencing the loss of beta-cell mass in islet transplantation. *Cell Transplant*. **2007**, 16, 1–8.
26. Buchwald, P. A local glucose-and oxygen concentration-based insulin secretion model for pancreatic islets. *Theor. Biol. Med. Model* **2011**, 8, 20.
27. Carlsson, P.O.; Liss, P.; Andersson, A.; Jansson, L. Measurements of oxygen tension in native and transplanted rat pancreatic islets. *Diabetes* **1998**, 47, 1027–1032.
28. Lai, Y.; Brandhorst, H.; Hossain, H.; Bierhaus, A.; Chen, C.; Bretzel, R.G.; Linn, T. Activation of NFκB dependent apoptotic pathway in pancreatic islet cells by hypoxia. *Islets* **2009**, 1, 19–25.
29. Wang, M.; Crager, M.; Pugazhenthi, S. Modulation of Apoptosis Pathways by Oxidative Stress and Autophagy in β Cells. *Exp. Diabetes Res*. **2012**, 2012, 647914.
30. Ryu, G.R.; Lee, M.K.; Lee, E.; Ko, S.H.; Ahn, Y.B.; Kim, J.W.; Yoon, K.H.; Song, K.H. Activation of AMP-activated protein kinase mediates strong and severe hypoxic injury to pancreatic beta cells. *Biochem. Biophys. Res. Commun*. **2009**, 386, 356–362.
31. Tian, L.; Kim, H.S.; Kim, H.; Jin, X.; Jung, H.S.; Park, K.S.; Cho, K.W.; Park, S.; Moon, W.K. Changes in metabolic markers in insulin-producing β-cells during hypoxia-induced cell death as studied by NMR metabolomics. *J. Proteome Res*. **2013**, 12, 3738–3745.
32. Qiao, N.; Xu, C.; Zhu, Y.-X.;, Cao, Y.; Liu, D.-C.; Han, X. Ets-1 as an early response gene against hypoxia-induced apoptosis in pancreatic β-cells. *Cell Death Dis*. **2015**, 6, e1650.

33. Polak, J.; Studer-Rabeler, K.; McHugh, H.; Hussain, M.A.; Shimoda, L.A. System for exposing cultured cells to intermittent hypoxia utilizing gas permeable cultureware. *Gen. Physiol. Biophys*. **2015**, 34, 235–247.
34. Nemcova-Fürstova, V.; Balusikova, K.; Sramek, J.; James, R.F.; Kovar, J. Caspase-2 and JNK Activated by Saturated Fatty Acids are Not Involved in Apoptosis Induction but Modulate ER Stress in Human Pancreatic β-cells. *Cell. Physiol. Biochem*. **2013**, 31, 277–289.
35. Sramek, J.; Nemcova-Fürstova, V.; Balusikova, K.; Daniel, P.; Jelinek, M.; James, R.F.; Kovar, J. p38 MAPK is activated but does not play a key role during apoptosis induction by saturated fatty Acid in human pancreatic β-cells. *Int. J. Mol. Sci*. **2016a**, 17, 159.
36. Sramek, J.; Nemcova-Fürstova, V.; Kovar, J. Kinase signaling in apoptosis induced by saturated fatty acids in pancreatic β-cells. *Int. J. Mol. Sci*. **2016b**, 17, 1400.
37. Sramek, J.; Nemcova-Fürstova, V.; Pavlikova, N.; Kovar, J. Effect of Saturated Stearic Acid on MAP Kinase and ER Stress Signaling Pathways during Apoptosis Induction in Human Pancreatic β-Cells Is Inhibited by Unsaturated Oleic Acid. *Int. J. Mol. Sci*. **2017**, 18, 2313.
38. Lagerstedt, S.A.; Hinrichs, D.R.; Batt, S.M.; Magera, M.J.; Rinaldo, P.; McConnell, J.P. Quantitative determination of plasma c8-c26 total fatty acids for the biochemical diagnosis of nutritional and metabolic disorders. *Mol. Genet. Metab*. **2001**, 73, 38–45.
39. Abdelmagid, S.A.; Clarke, S.E.; Nielsen, D.E.; Badawi, A.; El-Sohemy, A.; Mutch, D.M.; Ma, D.W.L. Comprehensive profiling of plasma fatty acid concentrations in young healthy Canadian adults. *PLoS ONE* **2015**, 10, e0116195.
40. Oslowski, C.M.; Urano, F. The binary switch that controls the life and death decisions of ER stressed beta cells. *Curr. Opin. Cell. Biol*. **2011**, 23, 207–215.
41. Tabas, I.; Ron, D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat. Cell Biol*. **2011**, 13, 184–190.
42. Semenza, G.L. Oxygen homeostasis. *Wiley Interdiscip. Rev. Syst. Biol. Med*. **2010**, 2, 336–361, 2010.
43. Chabowski, A.; Górski, J.; Calles-Escandon, J.; Tandon, N.N.; Bonen, A. Hypoxia-induced fatty acid transporter translocation increases fatty acid transport and contributes to lipid accumulation in the heart. *FEBS Lett*. **2006**, 580, 3617–3623.
44. Walford, G.A.; Moussignac, R.-L.; Scribner, A.W.; Loscalzoand, J.; Leopold, J.A. Hypoxia Potentiates Nitric Oxide-mediated Apoptosis in Endothelial Cells via Peroxynitrite-induced Activation of Mitochondria-dependent and -independent Pathways *J. Biol. Chem*. **2004**, 279, 4425–4432.
45. Bullova, P.; Cougnoux, A.; Abunimer, L.; Kopacek, J.; Pastorekova, S.; Pacak, K. Hypoxia potentiates the cytotoxic effect of piperlongumine in pheochromocytoma models. *Oncotarget* **2016**, 7, 40531–40545.
46. Dalgaard, L.T.; Thams, P.; Gaarn, L.W.; Jensen, J.; Lee, Y.C.; Nielsen, J.H. Suppression of FAT/CD36 mRNA by human growth hormone in pancreatic beta-cells. *Biochem. Biophys. Res. Commun*. **2011**, 410, 345–350.
47. Hyder, A.; Zenhom, M.; Klapper, M.; Herrmann, J.; Schrezenmeir, J. (2010) Expression of fatty acid binding proteins 3 and 5 genes in rat pancreatic islets and INS-1E cells: Regulation by fatty acids and glucose. *Islets* **2010**, 2, 174–184.

8. NEPUBLIKOVANÉ VÝSLEDKY A DALŠÍ VÝZKUM

Zjistili jsme, že proapoptotické působení SA (1 mM) zvyšuje fosforylaci EGFR receptoru (potenciálního členu jak p38 MAPK, tak ERK dráhy). OA (0,2 mM) je schopna inhibovat tento účinek SA a sama ovlivňuje fosforylaci EGFR výrazně méně než SA (obrázek 3). K podobným efektům vedla aplikace MK i v případě p38 MAPK signální dráhy (cf. publikace 4, obr. 2A, str. 96). Tyto výsledky tedy naznačují, že místo navození proapoptotické signalizace nasycenými MK, stejně jako místo intervence nenasycenými MK do mechanizmů indukce apoptózy nasycenými MK by se skutečně mohlo nalézat již na plazmatické membráně β-buněk, kde MK ovlivňují její fluiditu. Nasycené MK s rigidním a rovným acylovým řetězcem po inkorporaci do membránové dvojvrstvy snižují membránovou fluiditu. To může změnit schopnost membránových receptorových kináz, například EGFR, dimerizovat a tedy přenášet signály dále. Možný mechanizmus inhibiční intervence nenasycených MK v apoptotické signalizaci vyvolané nasycenými MK je již popsán v kapitole 6.2.4. Uvedené potenciální mechanizmy inhibiční intervence nenasycených MK, stejně jako mechanizmy, jakými dochází k navození proapoptotické signalizace nasycenými MK, bychom rádi ověřili specifickými experimenty. Tj., například měřením membránové fludity po aplikaci MK nebo zjišťováním zastoupení aplikovaných MK v plazmatické membráně. Také bychom chtěli objasnit, jakou konkrétní úlohu má EGFR v rámci mechanizmů indukce apoptózy nasycenými MK a mechanizmů inhibice této indukce nenasycenými MK. Dále máme k dispozici primární data ukazující na možné zapojení dalších molekul/signálních drah v rámci mechanizmů indukce apoptózy nasycenými MK. Jde o PKB signální dráhu, NF-κB signalizaci a další molekuly jako například některé STAT proteiny. Jejich konkrétní úloha v rámci uvedených mechanizmů by nás zajímala. Také bychom chtěli ověřit možné propojení některých těchto molekul s námi již studovanými signálními dráhami.

Dále bychom se chtěli zaměřit na objasnění molekulárních mechanizmů, kterými hypoxie zesiluje proapoptotický efekt SA a blokuje protektivní efekt OA. Rádi bychom našli místo intervence hypoxie do molekulárních mechanizmů indukce apoptózy a inhibice této indukce MK u pankreatických β-buněk. To se dle našich dosavadních výsledků musí nalézat „upstream“ nebo v rámci drah stresu ER. Kromě výše uvedeného bychom také chtěli přispět k objasnění molekulárních mechanizmů indukce apoptózy hypoxií samotnou. To zatím nebylo příliš studováno.



Obrázek 3. Efekt 1mM kyseliny stearové (SA), 1mM SA aplikované společně s 0.2mM kyselinou olejovou (OA), a 0.2mM OA na hladinu EGFR a fosfo-EGFR v NES2Y buňkách. Buňky inkubované bez MK představovaly kontrolní buňky. Po 3, 6, 12 a 24 hodinách inkubace byla zjištěna hladina EGFR proteinu western blot analýzou s použitím příslušných protilátek. Hladina aktinu byla zjišťována pro potvrzení stejného množství proteinů nanášených ve vzorcích. Prezentovaná data představují výsledek jednoho z nejméně tří nezávislých experimentů.

9. ZÁVĚRY

V této práci jsme se pokusili přispět k objasnění molekulárních mechanizmů indukce apoptózy nasycenými mastnými kyselinami (MK) a mechanizmů inhibice této indukce nenasycenými MK u lidských pankreatických β-buněk, a to na modelu buněk linie NES2Y. Prokázali jsme, že:

1. Aktivace kaspázy 2 působením nasycené kyseliny stearové (SA) v koncentraci indukující apoptózu (1 mM) není pro proces indukce apoptózy zásadní. Tato kaspáza však moduluje dráhy stresu endoplazmatického retikula (ER) indukované působením SA.
2. SA (1 mM) aktivuje p38 MAPK signální dráhu a inhibuje ERK signální dráhu. Inhibice ERK signální dráhy je pravděpodobně důsledkem aktivace p38 MAPK dráhy. Avšak p38 MAPK nejspíše není pro indukci apoptózy působením SA klíčová. Nenasycená kyselina olejová (OA, 0,2 mM) je schopná inhibovat výše uvedené účinky SA. Sama o sobě ovlivňuje aktivaci uvedených signálních drah pouze minimálně.
3. SA (1 mM) aktivuje dráhy stresu ER, tj. dráhy IRE1α, PERK a ATF6. OA (0,2 mM) je schopna inhibovat účinky SA na dráhy IRE1α a PERK a sama o sobě ovlivňuje aktivaci těchto drah pouze minimálně. Vliv OA na ATF6 dráhu nebyl zjišťován. JNK kináza, obdobně jako kaspáza 2, není pro proces indukce apoptózy navozené SA zásadní, ale moduluje dráhy stresu ER aktivované působením SA.
4. Místo navození proapoptotické signalizace nasycenými MK, stejně jako místo inhibiční intervence nenasycených MK do mechanizmů indukce apoptózy nasycenými MK, se nalézá „upstream“ od studovaných signálních drah. Pravděpodobně již na plazmatické membráně buněk.
5. Hypoxie zesiluje proapoptotický efekt SA (1 mM) pravděpodobně prostřednictvím zvýšení signalizace stresu ER. Hypoxie také snižuje protektivní efekt OA (0,2 mM) na proapoptotický účinek SA a to takovým způsobem, že OA již není schopna blokovat indukci apoptózy β-buněk vyvolanou působením SA. Hypoxie samotná má na β-buňky relativně slabý poškozující vliv. Hypoxie tedy může představovat klíčový faktor rozhodující o přežívání/smrti pankreatických β-buněk v přítomnosti MK a tedy v důsledku i o vzniku diabetu 2. typu (DMT2).

Naše výsledky přispěly k porozumění mechanizmů indukce apoptózy nasycenými MK a mechanizmů inhibice této indukce nenasycenými MK u pankreatických β-buněk.

Dále přispěly k porozumění mechanizmů vlivu hypoxie na indukci apoptózy nasycenými MK a na inhibici této indukce nenasycenými MK u β-buněk. Námi získaná data tedy v širším kontextu přispívají k pochopení mechanizmů, které hrají významnou úlohu v patogenezi DMT2. Zjištěné poznatky by mohly být užitečné při hledání nových možností diagnózy, léčby a prevence DMT2 a také při hledání nových možností zvýšení viability izolovaných Langerhansových ostrůvků pro transplantace. Protože stejné či obdobné molekulární mechanizmy regulace funkce a viability prostřednictvím MK se mohou uplatňovat i u jiných typů buněk (např. hepatocyty, kardiomyocyty), jsou zjištěné výsledky důležité i pro jiné oblasti medicíny (hepatologie či kardiologie).

10. SEZNAM POUŽITÉ LITERATURY

Abaraviciene SM, Lundquist I, Salehi A: Rosiglitazone counteracts palmitate-induced beta-cell dysfunction by suppression of MAP kinase, inducible nitric oxide synthase and caspase 3 activities. *Cell Mol Life Sci* 65(14): 2256-65, 2008.

Abdelmagid SA, Clarke SE, Nielsen DE, Badawi A, El-Sohemy A, Mutch DM, Ma DW: Comprehensive profiling of plasma fatty acid concentrations in young healthy Canadian adults. *PLoS One* 10(2): e0116195, 2015.

Acosta-Alvear D, Zhou Y, Blais A, Tsikitis M, Lents NH, Arias C, Lennon CJ, Kluger Y, Dynlacht BD: XBP1 controls diverse cell type- and condition-specific transcriptional regulatory networks. *Mol Cell* 27(1): 53-66, 2007.

Adams JM & Cory S: Apoptosomes: engines for caspase activation. *Curr Opin Cell Biol* 14(6): 715-20, 2002.

Afshar G, Jelluma N, Yang X, Basila D, Arvold ND, Karlsson A, Yount GL, Dansen TB, Koller E, Haas-Kogan DA: Radiation-induced caspase-8 mediates p53-independent apoptosis in glioma cells. *Cancer Res* 66(8): 4223-32, 2006.

Alejandro EU, Kalynyak TB, Taghizadeh F, Gwiazda KS, Rawstron EK, Jacob KJ, Johnson JD: Acute insulin signaling in pancreatic beta-cells is mediated by multiple Raf-1 dependent pathways. *Endocrinology* 151(2): 502-512, 2010.

Allagnat F, Cunha D, Moore F, Vanderwinden JM, Eizirik DL, Cardozo AK: Mcl-1 downregulation by pro-inflammatory cytokines and palmitate is an early event contributing to beta-cell apoptosis. *Cell Death Differ* 18(2): 328-37, 2011.

Appenzeller-Herzog C & Ellgaard L: The human PDI family: versatility packed into a single fold. *Biochim Biophys Acta* 1783(4): 535-48, 2008.

Araki E, Oyadomari S, Mori M: Endoplasmic reticulum stress and diabetes mellitus. *Intern Med* 42(1): 7-14, 2003.

Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB: Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130(1): 167-78, 1992.

Ashkenazi A & Dixit VM: Death receptors: signaling and modulation. *Science* 281(5381): 1305-8, 1998.

Bagrodia S, Derijard B, Davis RJ, Cerione RA: Cdc42 and PAKmediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. *J Biol Chem* 270(47): 27995-8, 1995.

Bachar E, Ariav Y, Ketzinel-Gilad M, Cerasi E, Kaiser N, Leibowitz G: Glucose amplifies fatty acid-induced endoplasmic reticulum stress in pancreatic beta-cells via activation of mTORC1. *PLoS One* 4(3): e4954, 2009.

Baldwin AC, Green CD, Olson LK, Moxley MA, Corbett JA: A role for aberrant protein palmitoylation in FFA-induced ER stress and beta cell death. *Am J Physiol Endocrinol Metab* 302(11): E1390-8, 2012.

Baliga BC, Read SH, Kumar S: The biochemical mechanism of caspase-2 activation. *Cell Death Differ* 11(11): 1234-41, 2004.

Barry M & Bleackley RC: Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol* 2(6): 401-9, 2002.

Beard J, Sladden T, Morgan G, Berry G, Brooks L, McMichael A: Health impacts of pesticide exposure in a cohort of outdoor workers. *Environ Health Perspect* 111(5): 724-30, 2003.

Bensellam M, Laybutt DR, Jonas JC: The molecular mechanisms of pancreatic β-cell glucotoxicity: recent findings and future research directions. *Mol Cell Endocrinol* 364(1-2): 1-27, 2012.

Bensellam M, Maxwell EL, Chan JY, Luzuriaga J, West PK, Jonas JC, Gunton JE, Laybutt DR: Hypoxia reduces ER-to-Golgi protein trafficking and increases cell death by inhibiting the adaptive unfolded protein response in mouse beta cells. *Diabetologia* 59(7): 1492-502, 2016.

Berra E, Diaz-Meco MT, Moscat J: The activation of p38 and apoptosis by the inhibition of ERK is antagonized by the phosphoinositide 3-kinase/Akt pathway. *J Biol Chem* 273(17): 10792-97, 1998.

Biden TJ, Boslem E, Chu KY, Sue N: Lipotoxic endoplasmic reticulum stress, β cell failure, and type 2 diabetes mellitus. *Trends Endocrinol Metab* 25(8): 389-98, 2014.

Bouchier-Hayes L & Green DR: Caspase-2: the orphan caspase. *Cell Death Differ* 19(1): 51-7, 2012.

Buchwald P: A local glucose-and oxygen concentration-based insulin secretion model for pancreatic islets. *Theor Biol Med Model* 8: 20, 2011.

Bullova P, Cougnoux A, Abunimer L, Kopacek J, Pastorekova S, Pacak K: Hypoxia potentiates the cytotoxic effect of piperlongumine in pheochromocytoma models. *Oncotarget* 7(26): 40531-45, 2016.

Bursch R: Multiple cell death programs: Charon's lifts to Hades. *FEMS Yeast Res* 5(2): 101-10, 2004.

Bursch W, Ellinger A, Gerner C, Frohwein U, Schulte-Hermann R: Programmed cell death (PCD). Apoptosis, autophagic PCD, or others? *Ann NY Acad Sci* 926: 1-12, 2000.

Busch AK, Gurisik E, Cordery DV, Sudlow M, Denyer GS, Laybutt DR, Hughes WE, Biden TJ: Increased fatty acid desaturation and enhanced expression of stearoyl coenzyme A desaturase protects pancreatic beta-cells from lipoapoptosis. *Diabetes* 54(10): 2917-24, 2005.

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC: Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* 52(1): 102-10, 2003.

Cagnol S & Chambard JC: ERK and cell death: mechanisms of ERK-induced cell death-apoptosis, autophagy and senescence. *FEBS J* 277(1): 2-21, 2009.

Cantley J, Grey ST, Maxwell PH, Withers DJ: The hypoxia response pathway and β-cell function. *Diabetes Obes Metab* 12(2): 159-67, 2010.

Cantley J, Selman C, Shukla D, Abramov AY, Forstreuter F, Esteban MA, Claret M, Lingard SJ, Clements M, Harten SK, Asare-Anane H, Batterham RL, Herrera PL, Persaud SJ, Duchen MR, Maxwell PH, Withers DJ: Deletion of the von Hippel-Lindau gene in pancreatic beta cells impairs glucose homeostasis in mice. *J Clin Invest* 119(1): 125-35, 2009.

Carlsson PO, Liss P, Andersson A, Jansson L: Measurements of oxygen tension in native and transplanted rat pancreatic islets. *Diabetes* 47(7): 1027-32, 1998

Carreau A, El Hafny-Rahbi B, Matejuk A, Grillon C, Kieda C: Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. *J Cell Mol Med* 15(6): 1239-53, 2011.

Cheung HH, Lynn KN, Liston P, Korneluk RG: Involvement of caspase-2 and caspase-9 in endoplasmic reticulum stress-induced apoptosis: a role for the IAPs. *Exp Cell Res* 312(12): 2347-57, 2006.

Cnop M, Hannaert JC, Hoorens A, Eizirik DL, Pipeleers DG: Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. *Diabetes* 50(8): 1771-7, 2001.

Cnop M, Hannaert JC, Pipeleers DG: Troglitazone does not protect rat pancreatic beta cells against free fatty acid-induced cytotoxicity. *Biochem Pharmacol* 63(7): 1281-5, 2002.

Cnop M, Igoillo-Esteve M, Cunha DA, Ladriere L, Eizirik DL: An update on lipotoxic endoplasmic reticulum stress in pancreatic beta-cells. *Biochem Soc Trans* 36(5): 909-15, 2008.

Cnop M, Ladriere L, Hekerman P, Ortis F, Cardozo AK, Dogusan Z, Flamez D, Boyce M, Yuan J, Eizirik DL: Selective inhibition of eukaryotic translation initiation factor 2 alpha dephosphorylation potentiates fatty acid-induced endoplasmic reticulum stress and causes pancreatic beta-cell dysfunction and apoptosis. *J Biol Chem* 282(6): 3989-97, 2007.

Cohen GM: Caspases: the executioners of apoptosis. *Biochem J* 326(1): 1-16, 1997.

Cohen P: The search for physiological substrates of MAP and SAP kinases in mammalian cells. *Trends Cell Biol* 7(9): 353-60, 1997.

Collombat P, Xu X, Heimberg H, Mansouri A: Pancreatic beta-cells: from generation to regeneration. *Semin Cell Dev Biol* 21(8): 838-44, 2010.

Corazza N, Jakob S, Schaer C., Frese S, Keogh A, Stroka D, Kassahn D, Torgler R, Mueller C, Schneider P, Brunner T: TRAIL receptor-mediated JNK activation and Bim phosphorylation critically regulate Fas-mediated liver damage and lethality. *J Clin Invest* 116(9): 2493-9, 2006.

Cornelis S, Bruynooghe Y, Van Loo G, Saelens X, Vandenabeele P, Beyaert R: Apoptosis of hematopoietic cells induced by growth factor withdrawal is associated with caspase-9 mediated cleavage of Raf-1. *Oncogene* 24(9): 1552-62, 2005.

Cory S & Adams JM: The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2(9): 647-56, 2002.

Cuenda A & Dorow DS: Differential activation of stress-activated protein kinase kinases SKK4/MKK7 and SKK1/MKK4 by the mixed-lineage kinase-2 and mitogen-activated protein kinase kinase (MKK) kinase-1. *Biochem J* 333(1): 11-5, 1998.

Cuenda A & Rousseau S: p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim Biophys Ac*ta 1773(8): 1358-75, 2007.

Cunha DA, Hekerman P, Ladriere L, Bazarra-Castro A, Ortis F, Wakeham MC, Moore F, Rasschaert J, Cardozo AK, Bellomo E, Overbergh L, Mathieu C, Lupi R, Hai T, Herchuelz A, Marchetti P, Rutter GA, Eizirik DL, Cnop M: Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. *J Cell Sci* 121(14): 2308-18, 2008.

Cunha DA, Ladriere L, Ortis F, Igoillo-Esteve M, Gurzov EN, Lupi R, Marchetti P, Eizirik DL, Cnop M: Glucagon-like peptide-1 agonists protect pancreatic beta-cells from lipotoxic endoplasmic reticulum stress through upregulation of BiP and JunB. *Diabetes* 58(12): 2851-62, 2009.

Cvjeticanin T, Stojanovic I, Timotijevic G, Stosic-Grujicic S, Miljkovic D: T cells cooperate with palmitic acid in induction of beta cell apoptosis. *BMC Immunol* 29: 1-10, 2009.

Dalgaard LT, Thams P, Gaarn LW, Jensen J, Lee YC, Nielsen JH: Suppression of FAT/CD36 mRNA by human growth hormone in pancreatic beta-cells. *Biochem Biophys Res Commun* 410(2): 345-50, 2011.

Darville MI & Eizirik DL: Regulation by cytokines of the inducible nitric oxide synthase promoter in insulin-producing cells. *Diabetologia* 41(9), 1101-8, 1998.

Debnath J, Baehrecke EH, Kroemer G: Does autophagy contribute to cell death? *Autophagy* 1(2): 66-74, 2005.

Deeney JT, Gromada J, Hoy M, Olsen HL, Rhodes CJ, Prentki M, Berggren PO, Corkey BE: Acute stimulation with long chain acyl-CoA enhances exocytosis in insulin-secreting cells (HIT T-15 and NMRI beta-cells). *J Biol Chem* 275(13): 9363-8, 2000.

Defronzo RA: Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* 58(4): 773-95, 2009.

Defronzo RA: Current issues in the treatment of type 2 diabetes. Overview of newer agents: where treatment is going. *Am J Med* 123(3): S38-48, 2010.

Degterev A, Boyce M, Yuan JY: A decade of caspases. *Oncogene* 22(53): 8543-67, 2003.

Deveraux QL, Takahashi R, Salvesen GS, Reed JC: X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* 388(6639): 300-4, 1997.

Devireddy LR, Teodoro JG, Richard FA, Green MR: Induction of apoptosis by a secreted lipocalin that is transcriptionally regulated by IL-3 deprivation. *Science* 293(5531): 829-34, 2001.

Dhayal S & Morgan NG: Structure-activity relationships influencing lipid-induced changes in eIF2alpha phosphorylation and cell viability in BRIN-BD11 cells. *FEBS Lett* 585(14): 2243-8, 2011.

Dhayal S, Welters HJ, Morgan NG: Structural requirements for the cytoprotective actions of mono-unsaturated fatty acids in the pancreatic beta-cell line, BRIN-BD11. *Br J Pharmacol* 153(8): 1718-27, 2008.

Dhillon AS, Meikle S, Yazici Z, Eulitz M, Kolch W: Regulation of Raf-1 activation and signaling by dephosphorylation. *EMBO J* 21(1-2): 64-71, 2002a.

Dhillon AS, Pollock C, Steen H, Shaw PE, Mischak H, Kolch W 2002. Cyclic AMP-dependent kinase regulates Raf-1 kinase mainly by phosphorylation of serine 259. *Mol Cell Biol* 22(10): 3237-46, 2002b.

Diakogiannaki E, Dhayal S, Childs CE, Calder PC, Welters HJ, Morgan NG: Mechanisms involved in the cytotoxic and cytoprotective actions of saturated versus monounsaturated long-chain fatty acids in pancreatic beta-cells. *J Endocrinol* 194(2): 283-91, 2007.

Diakogiannaki E, Welters HJ, Morgan NG: Differential regulation of the endoplasmic reticulum stress response in pancreatic beta-cells exposed to long-chain saturated and monounsaturated fatty acids. *J Endocrinol* 197(3): 553-63, 2008.

Diaz B, Barnard D, Filson A, MacDonald S, King A, Marshall M: Phosphorylation of Raf-1 serine 338-serine 339 is an essential regulatory event for Ras-dependent activation and biological signaling. *Mol Cell Biol* 17(8): 4509-16, 1997.

Donath MY, Ehses JA, Maedler K, Schumann DM, Ellingsgaard H, Eppler E, Reinecke M: Mechanisms of beta-cell death in type 2 diabetes. *Diabetes* 54(2): S108-13, 2005.

Droin N, Bichat F, Rebe C, Wotawa A, Sordet O, Hammann A, Bertrand R, Solary E: Involvement of caspase-2 long isoform in Fas-mediated cell death of human leukemic cells. *Blood* 97(6): 1835-44, 2001.

Du CY, Fang M, Li YC, Li L, Wang XD: Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell* 102(1): 33-42, 2000.

Ehrlichova M, Koc M, Truksa J, Nadova Z, Vaclavikova R, Kovar J: Cell death induced by taxanes in breast cancer cells: cytochrome C is released in resistant but not in sensitive cells. *Anticancer Res* 25(6B): 4215-24, 2005.

Eitel K, Staiger H, Brendel MD, Brandhorst D, Bretzel RG, Haring HU, Kellerer M: Different role of saturated and unsaturated fatty acids in beta-cell apoptosis. *Biochem Biophys Res Commun* 299(5): 853-6, 2002.

Eitel K, Staiger H, Rieger J, Mischak H, Brandhorst H, Brendel MD, Bretzel RG, Haring HU, Kellerer M: Protein kinase C delta activation and translocation to the nucleus are required for fatty acid-induced apoptosis of insulin-secreting cells. *Diabetes* 52(4): 991-7, 2003.

El Assaad W, Buteau J, Peyot ML, Nolan C, Roduit R, Hardy S, Joly E, Dbaibo G, Rosenberg L, Prentki M: Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death. *Endocrinology* 144(9): 4154-63, 2003.

Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol* 35(4): 495-516, 2007.

Emamaullee JA, Shapiro AM: Factors influencing the loss of beta-cell mass in islet transplantation. *Cell Transplant* 16(1): 1-8, 2007.

Enoksson M, Robertson JD, Gogvadze V, Bu P, Kropotov A, Zhivotovsky B, Orrenius S: Caspase-2 permeabilizes the outer mitochondrial membrane and disrupts the binding of cytochrome c to anionic phospholipids. *J Biol Chem* 279: 49575-8, 2004.

Fabian JR, Daar IO, Morrison DK: Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. *Mol Cell Biol* 13(11): 7170-9, 1993.

Fang Y, Zhang Q, Tan J, Li L, An X, Lei P: Intermittent hypoxia-induced rat pancreatic β-cell apoptosis and protective effects of antioxidant intervention. *Nutr Diabetes* 4: e131, 2014.

Fietta P: Many ways to die: passive and active cell death styles. *Riv Biol* 99(1): 69-83, 2006.

Fischer U, Janicke RU, Schulze-Osthoff K: Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ* 10(1): 76-100, 2003.

Fonseca SG, Ishigaki S, Oslowski CM, Lu S, Lipson KL, Ghosh R, Hayashi E, Ishihara H, Oka Y, Permutt MA, Urano F: Wolfram syndrome 1 gene negatively regulates ER stress signaling in rodent and human cells. *J Clin Invest* 120(3): 744-55, 2010.

Fonseca SG, Gromada J, Urano F: Endoplasmic reticulum stress and pancreatic beta-cell death. *Trends Endocrinol Metab* 22(7): 266-74, 2011.

Fontés G, Semache M, Hagman DK, Tremblay C, Shah R, Rhodes CJ, Rutter J, Poitout V: Involvement of Per-Arnt-Sim Kinase and extracellular-regulated kinases-1/2 in palmitate inhibition of insulin gene expression in pancreatic beta-cells. *Diabetes* 58(9): 2048-58, 2009.

Formigli L, Papucci L, Tani A, Schiavone N, Tempestini A, Orlandini GE, Capaccioli S, Orlandini SZ: Aponecrosis: morphological and biochemical exploration of a syncretic process of cell death sharing apoptosis and necrosis. *J Cell Physiol* 182(1): 41-9, 2000.

Fürstova V, Kopska T, James RF, Kovar J: Comparison of the effect of individual saturated and unsaturated fatty acids on cell growth and death induction in the human pancreatic β-cell line NES2Y. *Life Sci* 82(13-14): 684-691, 2008.

Garrido C, Galluzzi L, Brunet M, Puig PE, Didelot C, Kroemer G: Mechanisms of cytochrome c release from mitochondria. *Cell Death Differ* 13(9): 1423-33, 2006.

Georgiadi A & Kersten S: Mechanisms of gene regulation by fatty acids. *Adv Nutr* 3(2): 127-34, 2012.

Gerber PA & Rutter GA: The Role of Oxidative Stress and Hypoxia in Pancreatic Beta-Cell Dysfunction in Diabetes Mellitus. *Antioxid Redox Signal* 26(10): 501-18, 2016.

Glatz JF, Luiken JJ, Bonen A: Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. *Physiol Rev* 90(1): 367-417, 2010.

Greenhalgh DG: The role of apoptosis inwound healing. *Int J Biochem Cell Biol* 30(9): 1019-30, 1998.

Gu H, Chen X, Gao G, Dong H: Caspase-2 functions upstream of mitochondria in endoplasmic reticulum stress-induced apoptosis by bortezomib in human myeloma cells. *Mol Cancer Ther* 7(8): 2298-307, 2008.

Gunton JE, Kulkarni RN, Yim S, Okada T, Hawthorne WJ, Tseng YH, Roberson RS, Ricordi C, O'Connell PJ, Gonzalez FJ, Kahn CR: Loss of ARNT/HIF1beta mediates altered gene expression and pancreatic-islet dysfunction in human type 2 diabetes. *Cell* 122(3): 337-49, 2005.

Guo J, Qian YY, Xi XX, Hu XH, Zhu JX, Han X: Blockage of ceramide metabolism exacerbates palmitate inhibition of pro-insulin gene expression in pancreatic β-cells. *Mol Cell Biochem* 338(1-2): 283-90, 2010.

Gwiazda KS, Yang TL, Lin Y, Johnson JD: Effects of palmitate on ER and cytosolic Ca^{2+} homeostasis in beta-cells. *Am J Physiol Endocrinol Metab* 296(4): E690-701, 2009.

Haber EP, Procopio J, Carvalho CR, Carpinelli AR, Newsholme P, Curi R: New insights into fatty acid modulation of pancreatic beta-cell function. *Int Rev Cytol* 248: 1-41, 2006.

Haze K, Yoshida H, Yanagi H, Yura T, Mori K: Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol Biol Cell* 10(11): 3787-99, 1999.

He H, McColl K, Distelhorst CW: Involvement of c-Fos in signaling grp78 induction following ER calcium release. *Oncogene* 19(51): 5936-43, 2000.

Heinke S, Ludwig B, Schubert U, Schmid J, Kiss T, Steffen A, Bornstein S, Ludwig S: Diabetes induction by total pancreatectomy in minipigs with simultaneous splenectomy: a feasible approach for advanced diabetes research. *Xenotransplantation* 23(5): 405-13, 2016.

Hennige AM, Ranta F, Heinzelmann I, Dufer M, Michael D, Braumuller H, Lutz SZ, Lammers R, Drews G, Bosch F, Haring HU, Ullrich S: Overexpression of kinase-negative protein kinase Cdelta in pancreatic beta-cells protects mice from diet-induced glucose intolerance and beta-cell dysfunction. *Diabetes* 59(1): 119-27, 2010.

Hetz C, Martinon F, Rodriguez D, Glimcher LH: The unfolded protein response: integrating stress signals through the stress sensor IRE1alpha. *Physiol Rev* 91(4): 1219-43, 2011.

Hetz C: The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* 13(2): 89-102, 2012.

Higa M, Shimabukuro M, Shimajiri Y, Takasu N, Shinjyo T, Inaba T: Protein kinase B/Akt signalling is required for palmitate-induced beta-cell lipotoxicity. *Diabetes Obes Metab* 8(2): 228-33, 2006.

Hill MM, Adrain C, Duriez PJ, Creagh EM, Martin SJ: Analysis of the composition, assembly kinetics and activity of native Apaf-1 apoptosomes. *Embo J* 23(10): 2134-45, 2004.

Hirota N, Otabe S, Nakayama H, Yuan X, Yamada K: Sequential activation of caspases and synergistic beta-cell cytotoxicity by palmitate and anti-Fas antibodies. *Life Sci* 79(13): 1312-6, 2006.

Holzer RG, Park EJ, Li N, Tran H, Chen M, Choi C, Solinas G, Karin M: Saturated fatty acids induce c-Src clustering within membrane subdomains, leading to JNK activation. *Cell* 147(1): 173-84, 2011.

Huang CJ, Lin CY, Haataja L, Gurlo T, Butler AE, Rizza RA, Butler PC: High expression rates of human islet amyloid polypeptide induce endoplasmic reticulum stress mediated beta-cell apoptosis, a characteristic of humans with type 2 but not type 1 diabetes. *Diabetes* 56(8): 2016-27, 2007a.

Huang P, Akagawa K, Yokoyama Y, Nohara K, Kano K, Morimoto K: T-2 toxin initially activates caspase-2 and induces apoptosis in U937 cells. *Toxicol Lett* 170(1): 1-10, 2007b.

Huo J, Luo RH, Metz SA, Li G: Activation of caspase-2 mediates the apoptosis induced by GTP-depletion in insulin-secreting (HIT-T15) cells. *Endocrinology* 143(5): 1695-704, 2002.

Hyder A, Zenhom M, Klapper M, Herrmann J, Schrezenmeir J: Expression of fatty acid binding proteins 3 and 5 genes in rat pancreatic islets and INS-1E cells: Regulation by fatty acids and glucose. *Islets* 2(3): 174-84, 2010.

Chabowski A, Górski J, Calles-Escandon J, Tandon NN, Bonen A: Hypoxia-induced fatty acid transporter translocation increases fatty acid transport and contributes to lipid accumulation in the heart. *FEBS Lett* 580(15): 3617-23, 2006.

Chai W & Liu Z: p38 mitogen-activated protein kinase mediates palmitate-induced apoptosis but not inhibitor of nuclear factor-kappaB degradation in human coronary artery endothelial cells. *Endocrinology* 148(4): 1622-8, 2007.

Chang F, Steelman LS, Lee JT, Shelton JG, Navolanic PM, Blalock WL, Franklin RA, McCubrey JA: Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. *Leukemia* 17(7): 1263-93, 2003.

Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B. Wright A, Vanderbilt C, Cobb MH: MAP Kinases. *Chem Rev* 101(8): 2449-76, 2001.

Cheng K, Ho K, Stokes R, Scott C, Lau SM, Hawthorne WJ, O'Connell PJ, Loudovaris T, Kay TW, Kulkarni RN, Okada T, Wang XL, Yim SH, Shah Y, Grey ST, Biankin AV, Kench JG, Laybutt DR, Gonzalez FJ, Kahn CR, Gunton JE: Hypoxia-inducible factor-1alpha regulates beta cell function in mouse and human islets. *J Clin Invest* 120(6): 2171-83, 2010.

Cheung HH, Lynn KN, Liston P, Korneluk RG: Involvement of caäse-2 and caspase-9 in endoplasmic reticulum stress-induced apoptosis: a role for the IAPs. *Exp Cell Res* 312(12): 2347-57, 2006.

Chinnaiyan AM: The apoptosome: heart and soul of the cell death machine. *Neoplasia* 1(1): 5-15, 1999.

Choi SE, Kim HE, Shin HC, Jang HJ, Lee KW, Kim Y, Kang SS, Chun J, Kang Y: Involvement of Ca^{2+}-mediated apoptotic signals in palmitate-induced MIN6N8a beta cell death. *Mol Cell Endocrinol* 272(1-2): 50-62, 2007.

Christofferson DE & Yuan J: Necroptosis as an alternative form of programmed cell death. *Curr Opin Cell Biol* 22(2): 263-8, 2010.

Igney FH & Krammer PH: Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer* 2(4): 277-88, 2002.

Ichijo H, Nishida E, Irie K, ten Dijke P, Saitoh M, Moriguchi T, Takagi M, Matsumoto K, Miyazono K, Gotoh Y: Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* 275(5296): 90-4, 1997.

Ingalls ST, Xu Y, Hoppel CL: Determination of plasma non-esterified fatty acids and triglyceride fatty acids by gas chromatography of their methyl esters after isolation by column chromatography on silica gel. *J Chromatogr B Biomed Appl* 666(1): 1-12, 1995.

Inoue S, Browne G, Melino G, Cohen GM: Ordering of caspases in cells undergoing apoptosis by the intrinsic pathway. *Cell Death Differ* 16(7): 1053-61, 2009.

Ismail-Beigi F: Pathogenesis and glycemic management of type 2 diabetes mellitus: a physiological approach. *Arch Iran Med* 15(4): 239-46, 2012.

Jacobson MD, Weil M, Raff MC: Programmed cell death in animal development. *Cell* 88(3): 347-54, 1997.

Jeffrey KD, Alejandro EU, Luciani DS, Kalynyak TB, Hu X, Li H, Lin Y, Townsend RR, Polonsky KS, Johnson JD: Carboxypeptidase E mediates palmitate-induced beta-cell ER stress and apoptosis. *Proc Natl Acad Sci U S A* 105(24): 8452-7, 2008.

Jiang BH, Rue E, Wang GL, Roe R, Semenza GL: Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *J Biol Chem* 271(30): 17771-8, 1996.

Jiang Y, Chen C, Li Z, Guo W, Gegner JA, Lin S, Han J: Characterization of the structure and function of a new mitogen-activated protein kinase (p38b). *J Biol Chem* 271(30): 17920-6, 1996.

Jiang Y, Gram H, Zhao M, New L, Gu J, Feng L, Di Padova F, Ulevitch RJ, Han J: Characterization of the structure and function of the fourth member of p38 group mitogenactivated protein kinases, p38delta. *J Biol Chem* 272(48): 30122-8, 1997.

Jitrapakdee S, Wutthisathapornchai A, Wallace JC, MacDonald MJ: Regulation of insulin secretion: role of mitochondrial signalling. *Diabetologia* 53(6): 1019-32, 2010.

Johnson JD, Han Z, Otani K, Ye H, Zhang Y, Wu H, Horikawa Y, Misler S, Bell GI, Polonsky KS: RyR2 and calpain-10 delineate a novel apoptosis pathway in pancreatic islets. *J Biol Chem* 279(23): 24794-802, 2004.

Jonas JC, Sharma A, Hasenkamp W, Ilkova H, Patanè G, Laybutt R, Bonner-Weir S, Weir GC: Chronic hyperglycemia triggers loss of pancreatic b cell differentiation in an animal model of diabetes. *J Biol Chem* 274(20): 14112-21, 1999.

Joseph JW, Koshkin V, Zhang CY, Wang J, Lowell BB, Chan CB, Wheeler MB: Uncoupling protein 2 knockout mice have enhanced insulin secretory capacity after a high-fat diet. *Diabetes* 51(11): 3211-9, 2002.

Jump DB: Fatty acid regulation of gene transcription. *Crit Rev Clin Lab Sci* 41(1): 41-78, 2004.

Kadowaki T: Insights into insulin resistance and type 2 diabetes from knockout mouse models. *J Clin Invest* 106(4): 459-65, 2000.

Kahn SE, Prigeon RL, McCulloch DK, Boyko EJ, Bergman RN, Schwartz MW, Neifing JL, Ward WK, Beard JC, Palmer JP: Quantification of the relationship between insulin sensitivity and beta-cell function in human subjects. Evidence for a hyperbolic function. *Diabetes* 42(11): 1663-72, 1993.

Kahn SE, Hull RL, Utzschneider KM: Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 444(7121): 840-6, 2006.

Kallunki T, Deng T, Hibi M, Karin M: c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. *Cell* 87(5): 929-39, 1996.

Kamp F & Hamilton JA: How fatty acids of different chain length enter and leave cells by free diffusion. *Prostaglandins Leukot Essent Fatty Acids* 75(3): 149-59, 2006.

Kaneko Y, Ishikawa T, Amano S, Nakayama K: Dual effect of nitric oxide on cytosolic Ca^{2+} concentration and insulin secretion in rat pancreatic beta-cells. *Am J Physiol Cell Physiol* 284(5), C1215-22, 2003.

Kaneto H, Fujii J, Seo HG, Suzuki K, Matsuoka T, Nakamura M, Tatsumi H, Yamasaki Y, Kamada T, Taniguchi N: Apoptotic cell death triggered by nitric oxide in pancreatic β-cells. *Diabetes* 44: 733-8, 1995.

Kang KA, Lee KH, Kim SY, Kim HS, Kim JS, Hyun JW: Cytoprotective effects of KIOM-79 on streptozotocin induced cell damage by inhibiting ERK and AP-1. *Biol Pharm Bull* 30(5): 852-8, 2007.

Karaskov E, Scott C, Zhang L, Teodoro T, Ravazzola M, Volchuk A: Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis. *Endocrinology* 147(7): 3398-407, 2006.

Karnovsky MJ, Kleinfeld AM, Hoover RL, Klausner RD: The concept of lipid domains in membranes. *J Cell Biol* 94(1): 1-6, 1982.

Kataoka T, Schroter M, Hahne M, Schneider P, Irmler M, Thome M, Froelich CJ, Tschopp J: FLIP prevents apoptosis induced by death receptors but not by perforin/granzyme B, chemotherapeutic drugs, and gamma irradiation. *J Immunol* 161(8): 3936-42, 1998.

Kerr JF, Wyllie AH, Currie AR: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26(4): 239-57, 1972.

Kharroubi I, Ladriere L, Cardozo AK, Dogusan Z, Cnop M, Eizirik DL: Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress. *Endocrinology* 145(11): 5087-96, 2004.

Kim IR, Murakami K, Chen NJ, Saibil SD, Matysiak-Zablocki E, Elford AR, Bonnard M, Benchimol S, Jurisicova A, Yeh WC, Ohashi PS: DNA damage- and stress-induced apoptosis occurs independently of PIDD. *Apoptosis* 14(9): 1039-49, 2009.

Kim JW, Tchernyshyov I, Semenza GL, Dang CV: HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab* 3(3): 177-85, 2006.

Kim K, Chung MH, Park S, Cha J, Baek JH, Lee SY, Choi SY: ER stress attenuation by Aloe-derived polysaccharides in the protection of pancreatic β-cells from free fatty acid-induced lipotoxicity. *Biochem Biophys Res Commun* 500(3): 797-803, 2018.

Kim TK, Lee JS, Jung HS, Ha TK, Kim SM, Han N, Lee EJ, Kim TN, Kwon MJ, Lee SH, Kim MK, Rhee BD, Park JH: Triiodothyronine induces proliferation of pancreatic β-cells through the MAPK/ERK pathway. *Exp Clin Endocrinol Diabetes* 122(4): 240-5, 2014.

Kinchen JM, Hengartner MO: Tales of cannibalism, suicide, and murder: Programmed cell death in C. elegans. *Curr Top Dev Biol* 65: 1-45, 2005.

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME: Cytotoxicitydependent APO-1 (Fas/CD95)- associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J* 14(22): 5579-88, 1995.

Ko SH, Ryu GR, Kim S, Ahn YB, Yoon KH, Kaneto H, Ha H, Kim YS, Song KH: Inducible nitric oxide synthase-nitric oxide plays an important role in acute and severe hypoxic injury to pancreatic beta cells. *Transplantation* 85(3): 323-30, 2008.

Koc M, Nad'ová Z, Kovář J: Sensitivity of cells to apoptosis induced by iron deprivation can be reversibly changed by iron availability. *Cell Prolif* 39(6): 551-61, 2006.

Koc M, Nad'ova Z, Truksa J, Ehrlichova M, Kovar J: Iron deprivation induces apoptosis via mitochondrial changes related to Bax translocation. *Apoptosis* 10(2): 381-93, 2005.

Kojima I & Umezawa K: Conophylline: a novel differentiation inducer for pancreatic beta cells. *Int J Biochem Cell Biol* 38(5-6): 923-30, 2006.

Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marmé D, Rapp UR: Protein kinase Ca activates RAF-1 by direct phosphorylation. *Nature* 364(6434): 249-52, 1993.

Koul D, Takada Y, Shen R, Aggarwal BB, Yung WK: PTEN enhances TNF-induced apoptosis through modulation of nuclear factor-kappaB signaling pathway in human glioma cells. *Biochem Biophys Res Commun* 350(2): 463-71, 2006.

Kovář J & Franěk F: Growth-stimulating effect of transferrin on a hybridoma cell line: relation to transferrin iron-transporting function. *Exp Cell Res* 182(2): 358-69, 1989.

Kralova J, Synytsya A, Pouckova P, Koc M, Dvorak M, Kral V: Novel porphyrin conjugates with a potent photodynamic antitumor effect: differential efficacy of mono- and bis-beta-cyclodextrin derivatives in vitro and in vivo. *Photochem Photobiol* 82(2): 432-38, 2006.

Kulkarni RN: The islet beta-cell. *Int J Biochem Cell Biol* 36(3): 365-71, 2004.

Kumar S, McDonnell PC, Gum RJ, Hand AT, Lee JC, Young PR: Novel homologues of CSBP/p38 MAP kinase: activation, substrate specificity and sensitivity to inhibition by pyridinyl imidazoles. *Biochem Biophys Res Commun* 235(3): 533-8, 1997.

Kumar S: Caspase function in programmed cell death. *Cell Death Differ* 14(1): 32-43, 2007.

Kutlu B, Cardozo AK, Darville MI, Kruhøffer M, Magnusson N, Øntoft T, Eizirik DL: Discovery of gene networks regulating cytokine-induced dysfunction and apoptosis in insulin-producing INS-1 cells. *Diabetes* 52(11), 2701–2719, 2003.

Kyriakis JM & Avruch J: Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* 81(2): 807-69, 2001.

Ladriere L, Igoillo-Esteve M, Cunha DA, Brion JP, Bugliani M, Marchetti P, Eizirik DL, Cnop M: Enhanced signaling downstream of ribonucleic Acid-activated protein kinase-like endoplasmic reticulum kinase potentiates lipotoxic endoplasmic reticulum stress in human islets. *J Clin Endocrinol Metab* 95(3), 1442-9, 2010.

Lagerstedt SA, Hinrichs DR, Batt SM, Magera MJ, Rinaldo P, McConnell JP: Quantitative determination of plasma c8-c26 total fatty acids for the biochemical diagnosis of nutritional and metabolic disorders. *Mol Genet Metab* 73(1): 38-45, 2001.

Lai E, Bikopoulos G, Wheeler MB, Rozakis-Adcock M, Volchuk A: Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic beta-cells. *Am J Physiol Endocrinol Metab* 294(3): E540-50, 2008.

Lai Y, Brandhorst H, Hossain H, Bierhaus A, Chen C, Bretzel RG, Linn T: Activation of NFκB dependent apoptotic pathway in pancreatic islet cells by hypoxia. *Islets* 1(1): 19-25, 2009.

Lang F, Ullrich S, Gulbins E: Ceramide formation as a target in beta-cell survival and function. *Expert Opin Ther Targets* 15(9): 1061-71, 2011.

Lassus P, Opitz-Araya X, Lazebnik Y: Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* 297(5585): 1352-4, 2002.

Lavrik IN, Golks A, Baumann S, Krammer PH: Caspase-2 is activated at the CD95 death-inducing signaling complex in the course of CD95-induced apoptosis. *Blood* 108(2): 559-65, 2006.

Laybutt DR, Preston AM, Akerfeldt MC, Kench JG, Busch AK, Biankin AV, Biden TJ: Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. *Diabetologia* 50(4): 752-63, 2007.

Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM: Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 379(6566): 632-5, 1996.

Lee J, Hong F, Kwon S, Kim SS, Kim DO, Kang HS, Lee SJ, Ha J, Kim SS: Activation of p38MAPK induces cell cycle arrest via inhibition of c-Raf/ERK pathway during muscle differentiation. *Biochem Biophys Res Commun* 298(5): 765-71, 2002.

Legrand P & Rioux V: The complex and important cellular and metabolic functions of saturated fatty acids. *Lipids* 45(10): 941-6, 2010.

Lechner C, Zahalka MA, Giot JF, Moler NPH, Ullrich A: ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation. *Proc Natl Acad Sci USA* 93(9): 4355-9, 1996.

Leung KT, Li KK, Sun SS, Chan PK, Ooi VE, Chiu LC: Activation of the JNK pathway promotes phosphorylation and degradation of BimEL – a novel mechanism of chemoresistance in T-cell acute lymphoblastic leukemia. *Carcinogenesis* 29(3): 544-51, 2008.

Li J, Liu X, Ran X, Chen J, Li X, Wu W, Huang H, Huang H, Long Y, Liang J, Cheng J, Tian H: Sterol regulatory element-binding protein-1c knockdown protected INS-1E cells from lipotoxicity. *Diabetes Obes Metab* 12(1): 35-46, 2010.

Li X, Zhang L, Meshinchi S Dias-Leme C, Raffin D, Johnson JD, Treutelaar MK, Burant CF: Islet microvasculature in islet hyperplasia and failure in a model of type 2 diabetes. *Diabetes* 55(11): 2965-73, 2006.

Li Z, Jiang Y, Ulevitch RJ, Han J: The primary structure of p38gamma: A new member of p38 group of MAP kinase. *Biochem Biophys Res Comm* 228(2): 334-40, 1996.

Liadis N, Salmena L, Kwan E, Tajmir P, Schroer SA, Radziszewska A, Li X, Sheu L, Eweida M, Xu S, Gaisano HY, Hakem R, Woo M: Distinct in vivo roles of caspase-8 in beta-cells in physiological and diabetes models. *Diabetes* 56(9): 2302-11, 2007.

Lieberman J & Fan Z: Nuclear war: the granzyme A-bomb. *Curr Opin Immunol* 15(5): 553-9, 2003.

Lin N, Chen H, Zhang H, Wan X, Su Q: Mitochondrial reactive oxygen species (ROS) inhibition ameliorates palmitate-induced INS-1 beta cell death. *Endocrine* 42(1): 107-17, 2012.

Liu J, Chang F, Li F, Fu H, Wang J, Zhang S, Zhao J, Yin D: Palmitate promotes autophagy and apoptosis through ROS-dependent JNK and p38 MAPK. *Biochem Biophys Res Commun* 463(3): 262-267, 2015.

Liu W, Shen S-M, Zhao X-Y, Chen G-Q: Targeted genes and interacting proteins of hypoxia inducible factor-1. *Int J Biochem Mol Biol* 3(2): 165-78, 2012.

Liu Z & Cao W: p38 mitogen-activated protein kinase: a critical node linking insulin resistance and cardiovascular diseases in type 2 diabetes mellitus. *Endocr Metab Immune Disord Drug Targets* 9(1): 38-46, 2009.

Lock LT, Laychock SG, Tzanakakis ES: Pseudoislets in stirred-suspension culture exhibit enhanced cell survival, propagation and insulin secretion. *J Biotechnol* 151(3): 278-86, 2011.

Logette E, Le Jossic-Corcos C, Masson D, Solier S, Sequeira-Legrand A, Dugail I, Lemaire-Ewing S, Desoche L, Solary E, Corcos L: Caspase-2, a novel lipid sensor under the control of sterol regulatory element binding protein 2. *Mol Cell Biol* 25(21): 9621-31, 2005.

Loo G, Gurp M, Depuydt B, Srinivasula SM, Rodriguez I, Alnemri ES, Gevaert K, Vandekerckhove J, Declercq W, Vandenabeele P: The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. *Cell Death Differ* 9(1): 20-6, 2002.

Lu X, Liu J, Hou F, Liu Z, Cao X, Seo H, Gao B: Cholesterol induces pancreatic β cell apoptosis through oxidative stress pathway. *Cell Stress Chaperones* 16(5): 539-48, 2011.

Lund LR, Romer J, Thomasset N, Solberg H, Pyke C, Bissell MJ, Dano K, Werb Z. Two distinct phases of apoptosis in mammary gland involution: proteinase-independent and -dependent pathways. *Development* 122: 181-93, 1996.

Lupi R, Dotta F, Marselli L, Del Guerra S, Masini M, Santangelo C, Patane G, Boggi U, Piro S, Anello M, Bergamini E, Mosca F, Di Mario U, Del Prato S, Marchetti P: Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes* 51(5): 1437-42, 2002.

Ma K, Nunemaker CS, Wu R, Chakrabarti SK, Taylor-Fishwick DA, Nadler JL: 12-Lipoxygenase Products Reduce Insulin Secretion and β-Cell Viability in Human Islets. *J Clin Endocrinol Metab* 95(2): 887-93, 2010.

Ma Y & Hendershot LM: Delineation of a negative feedback regulatory loop that controls protein translation during endoplasmic reticulum stress. *J Biol Chem* 278(37): 34864-73, 2003.

Ma Z, Moruzzi N, Catrina SB, Grill V, Björklund A: Hyperoxia inhibits glucose-induced insulin secretion and mitochondrial metabolism in rat pancreatic islets. *Biochem Biophys Res Commun* 443(1): 223-8, 2014.

Macfarlane WM, Cragg H, Docherty HM, Read ML, James RF, Aynsley-Green A, Docherty K: Impaired expression of transcription factor IUF1 in a pancreatic beta-cell line derived from a patient with persistent hyperinsulinaemic hypoglycaemia of infancy (nesidioblastosis). *FEBS Lett* 413(2): 304-8, 1997.

Maedler K, Spinas GA, Lehmann R, Sergeev P, Weber M, Fontana A, Kaiser N, Donath MY: Glucose induces beta-cell apoptosis via upregulation of the Fas receptor in human islets. *Diabetes* 50(8): 1683-90, 2001.

Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, Spinas GA, Kaiser N, Halban PA, Donath MY: Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. *J Clin Invest* 110(6): 851-60, 2002.

Maedler K, Oberholzer J, Bucher P, Spinas GA, Donath MY: Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. *Diabetes* 52(3): 726-33, 2003.

Maedler K: Beta cells in type 2 diabetes – a crucial contribution to pathogenesis. *Diabetes Obes Metab* 10(5): 408-20, 2008.

Maestre I, Jordan J, Calvo S, Reig JA, Cena V, Soria B, Prentki M, Roche E: Mitochondrial dysfunction is involved in apoptosis induced by serum withdrawal and fatty acids in the beta-cell line INS-1. *Endocrinology* 144(1): 335-45, 2003.

Makeeva N, Myers JW, Welsh N: Role of MKK3 and p38 MAPK in cytokine-induced death of insulin-producing cells. *Biochem J* 393(1): 129-39, 2006.

Mansilla S, Priebe W, Portugal J: Mitotic catastrophe results in cell death by caspase-dependent and caspase-independent mechanisms. *Cell Cycle* 5(1): 53-60, 2006.

Manzl C, Krumschnabel G, Bock F, Sohm B, Labi V, Baumgartner F, Logette E, Tschopp J, Villunger A: Caspase-2 activation in the absence of PIDDosome formation. *J Cell Biol* 185(2): 291-303, 2009.

Marchetti P, Bugliani M, Lupi R, Marselli L, Masini M, Boggi U, Filipponi F, Weir GC, Eizirik DL, Cnop M: The endoplasmic reticulum in pancreatic beta cells of type 2 diabetes patients. *Diabetologia* 50(12): 2486-94, 2007.

Marchetti P, Del GS, Marselli L, Lupi R, Masini M, Pollera M, Bugliani M, Boggi U, Vistoli F, Mosca F, Del PS: Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin. *J Clin Endocrinol Metab* 89(11): 5535-41, 2004.

Martin DA, Siegel RM, Zheng LX, Lenardo MJ: Membrane oligomerization and cleavage activates the caspase-8 (FLICE/MACH alpha 1) death signal. *J Biol Chem* 273: 4345-9, 1998.

Matsubara H, Mizutani Y, Hongo F, Nakanishi H, Kimura Y, Ushijima S, Kawauchi A, Tamura T, Sakata T, Miki T: Gene therapy with TRAIL against renal cell carcinoma. *Mol Cancer Ther* 5(9): 2165-71, 2006.

Matsuyama W, Faure M, Yoshimura T: Activation of discoidin domain receptor 1 facilitates the maturation of human monocyte-derived dendritic cells through the TNF receptor associated factor 6/TGF-beta-activated protein kinase 1 binding protein 1 beta/p38 alpha mitogen-activated protein kinase signaling cascade. *J Immunol* 171(7): 3520-32, 2003.

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ: The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 399(6733): 271-5, 1999.

Meier JJ, Butler AE, Saisho Y, Monchamp T, Galasso R, Bhushan A, Rizza RA, Butler PC: Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. *Diabetes* 57(6): 1584-94, 2008.

Meier P, Finch A, Evan G. Apoptosis in development. *Nature* 407(6805): 796-801, 2000.

Miao G, Ostrowski RP, Mace J, Hough J, Hopper A, Peverini R, Chinnock R, Zhang J, Hathout E: Dynamic production of hypoxia-inducible factor-1a in early transplanted islets. *Am J Transplant* 6(11): 2636-43, 2006.

Moffitt JH, Fielding BA, Evershed R, Berstan R, Currie JM, Clark A: Adverse physicochemical properties of tripalmitin in beta cells lead to morphological changes and lipotoxicity in vitro. *Diabetologia* 48(9): 1819-29, 2005.

Morgan NG & Dhayal S: G-protein coupled receptors mediating long chain fatty acid signalling in the pancreatic beta-cell. *Biochem Pharmacol* 78(12): 1419-27, 2009.

Moriguchi T, Kuroyanagi N, Yamaguchi K, Gotoh Y, Irie K, Kano T, Shirakabe K, Muro Y, Shibuya H, Matsumoto K, Nishida E, Hagiwara M: A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3. *J Biol Chem* 271(23): 13675-9, 1996.

Moritz W, Meier F, Stroka DM, Giuliani M, Kugelmeier P, Nett PC, Lehmann R, Candinas D, Gassmann M, Weber M: Apoptosis in hypoxic human pancreatic islets correlates with HIF-1alpha expression. *FASEB J* 16(7): 745-7, 2002.

Natalicchio A, Labarbuta R, Tortosa F, Biondi G, Marrano N, Peschechera A, Carchia E, Orlando MR, Leonardini A, Cignarelli A, Marchetti P, Perrini S, Laviola L, Giorgino F: Exendin-4 protects pancreatic beta cells from palmitate-induced apoptosis by interfering with GPR40 and the MKK4/7 stress kinase signalling pathway. *Diabetologia* 56(11): 2456-66, 2013.

Němcová-Fürstová V, James RFL, Kovář J: Inhibitory effect of unsaturated fatty acids on saturated fatty acids-induced apoptosis in human pancreatic β-cells: activation of caspases and ER stress induction. *Cell Physiol Biochem* 27(5): 525-38, 2011.

Němcová-Fürstová V, Šrámek J, Balušíková K, James RF, Kovář J: Caspase-2 and JNK activated by saturated fatty acids are not involved in apoptosis induction but modulate ER stress in human pancreatic β-cells. *Cell Physiol Biochem* 31(2-3): 277-89, 2013.

Newsholme P, Keane D, Welters HJ, Morgan NG: Life and death decisions of the pancreatic beta-cell: the role of fatty acids. *Clin Sci (Lond)* 112(1): 27-42, 2007.

Nijhawan D, Honarpour N, Wang X: Apoptosis in neural development and disease. *Annu Rev Neurosci* 23: 73-87, 2000.

Nikolova-Karakashian MN & Rozenova KA: Ceramide in stress response. *Adv Exp Med Biol* 688: 86-108, 2010.

Nishitoh H: CHOP is a multifunctional transcription factor in the ER stress response. *J Biochem* 151(3): 217-9, 2012.

Noguchi A, Takada M, Nakayama K, Ishikawa T: cGMP-independent anti-apoptotic effect of nitric oxide on thapsigargin-induced apoptosis in the pancreatic beta-cell line INS-1. *Life Sci* 83(25-26): 865-70, 2008.

Noushmehr H, D'Amico E, Farilla L, Hui H, Wawrowsky KA, Mlynarski W, Doria A, Abumrad NA, Perfetti R: Fatty acid translocase (FAT/CD36) is localized on insulin-containing granules in human pancreatic beta-cells and mediates fatty acid effects on insulin secretion. *Diabetes* 54(2): 472-81, 2005.

Okuyama R, Fujiwara T, Ohsumi J: High glucose potentiates palmitate-induced NO-mediated cytotoxicity through generation of superoxide in clonal beta-cell HIT-T15. *FEBS Lett* 545(2-3): 219-23, 2003.

Olsson M, Vakifahmetoglu H, Abruzzo PM, Hogstrand K, Grandien A, Zhivotovsky B: DISC-mediated activation of caspase-2 in DNA damage-induced apoptosis. *Oncogene* 28(18): 1949-59, 2009.

Olsson R, Olerud J, Pettersson U, Carlsson PO: Increased numbers of low-oxygenated pancreatic islets after intraportal islet transplantation. *Diabetes* 60(9): 2350-3, 2011.

Opferman JT, Korsmeyer SJ: Apoptosis in the development and maintenance of the immune system. *Nat Immunol* 4(5): 410-5, 2003.

Oslowski CM, Urano F: The binary switch that controls the life and death decisions of ER stressed beta cells. *Curr Opin Cell Biol* 23(2): 207-15, 2011.

Ou D, Wang X, Metzger DL, James RF, Pozzilli P, Plesner A, Korneluk RG, Verchere CB, Tingle AJ: Synergistic inhibition of tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human pancreatic beta cells by Bcl-2 and X-linked inhibitor of apoptosis. *Hum Immunol* 66(3): 274-84, 2005a.

Ou D, Wang X, Metzger DL, Robbins M, Huang J, Jobin C, Chantler JK, James RF, Pozzilli P, Tingle AJ: Regulation of TNF-related apoptosis-inducing ligand-mediated death-signal pathway in human beta cells by Fas- associated death domain and nuclear factor kappaB. *Hum Immunol* 66(7): 799-809, 2005b.

Oyadomari S, Koizumi A, Takeda K, Gotoh T, Akira S, Araki E, Mori M: Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes. *J Clin Invest* 109(4): 525-32, 2002.

Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC: HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metab* 3(3): 187-97, 2006.

Park BD, Ham YM, Jeong HJ, Cho SJ, Je YT, Yoo KD, Lee SK: Phosphorylation of Smac by JNK3 attenuates its interaction with XIAP. *Biochem Biophys Res Commun* 361(4): 994-9, 2007.

Park J, Kim I, Oh YJ, Lee K, Han PL, Choi EJ: Activation of c-Jun N-terminal kinase antagonizes an anti-apoptotic action of Bcl-2. *J Biol Chem* 272(27): 16725-8, 1997.

Parnaud G, Hammar E, Ribaux P, Donath MY, Berney T, Halban PA: Signaling pathways implicated in the stimulation of beta-cell proliferation by extracellular matrix. *Mol Endocrinol* 23(8): 1264-71, 2009.

Pedraza E, Coronel MM, fraker CA, Ricordi C, Stabler CL: Preventing hypoxia-induced cell death in beta cells and islets via hydrolytically activated, oxygen-generating biomaterials. *PNAS* 109(11): 4245-50, 2012.

Phillips MS, Liu Q, Hammond HA, Dugan V, Hey PJ, Caskey CJ, Hess JF: Leptin receptor missense mutation in the fatty Zucker rat. *Nat Genet* 13(1): 18-9, 1996.

Piacentini M, Evangelisti C, Mastroberardino PG, Nardacci R, Kroemer G: Does prothymosin-alpha act as molecular switch between apoptosis and autophagy? *Cell Death Differ* 10(9): 937-9, 2003.

Pirot P, Ortis F, Cnop M, Ma Y, Hendershot LM, Eizirik DL, Cardozo AK: Transcriptional regulation of the endoplasmic reticulum stress gene chop in pancreatic insulin-producing cells. *Diabetes* 56(4): 1069-77, 2007.

Plaisance V, Perret V, Favrea D, Abderrahmania A, Yanga J-Y, Widmanna C, Regazzi R: Role of the transcriptional factor C/EBP in free fatty acid-elicited beta cell failure. *Mol Cell Endocrinol* 305(1-2): 47-55, 2009.

Poitout V, Amyot J, Semache M, Zarrouki B, Hagman D, Fontes G: Glucolipotoxicity of the pancreatic beta cell. *Biochim Biophys Acta* 1801(3): 289-98, 2010.

Pop C & Salvesen GS: Human Caspases: Activation, Specificity, and Regulation. *J Biol Chem* 284(33): 21777-81, 2009.

Pouysségur J, Volmat V, Lenormand, P: Fidelity and spatio-temporal control in MAP kinase (ERKs) signaling. *Biochem Pharmacol* 64(5-6): 755-63, 2002.

Pratley RE & Weyer C: The role of impaired early insulin secretion in the pathogenesis of Type II diabetes mellitus. *Diabetologia* 44(8): 929-45, 2001.

Puri S, Cano DA, Hebrok M: A Role for von Hippel-Lindau Protein in Pancreatic β-Cell Function. Diabetes 58(2): 433-41, 2009.

Qiao N, Xu C, Zhu1 Y-X, Cao Y, Liu D-C, Han X: Ets-1 as an early response gene against hypoxia-induced apoptosis in pancreatic β-cells. *Cell Death Dis* 6: e1650, 2015.

Rai NK, Tripathi K, Sharma D, Shukla VK: Apoptosis: a basic physiologic process in wound healing. *Int J Low Extrem Wounds* 4(3): 138-44, 2005.

Rakatzi I, Mueller H, Ritzeler O, Tennagels N, Eckel J: Adiponectin counteracts cytokine- and fatty acid-induced apoptosis in the pancreatic beta-cell line INS-1. *Diabetologia* 47(2): 249-58, 2004.

Ramachandran S, Desai NM, Goers TA, Benshoff N, Olack B, Shenoy S, Jendrisak MD, Chapman WC, Mohanakumar T: Improved islet yields from pancreas preserved in perflurocarbon is via inhibition of apoptosis mediated by mitochondrial pathway. *Am J Transplant* 6(7): 1696-703, 2006.

Reusch HP, Zimmermann S, Schaefer M, Paul M, Moelling K: Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells. *J Biol Chem* 276(36): 33630-37, 2001.

Ritzel RA, Meier JJ, Lin CY, Veldhuis JD, Butler PC: Human islet amyloid polypeptide oligomers disrupt cell coupling, induce apoptosis, and impair insulin secretion in isolated human islets. *Diabetes* 56(1): 65-71, 2007.

Rodriguez D, Rojas-Rivera D, Hetz C: Integrating stress signals at the endoplasmic reticulum: The BCL-2 protein family rheostat. *Biochim Biophys Acta* 1813(4): 564-74, 2011.

Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC: The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J* 16(23): 6914-25, 1997.

Ryu GR, Lee MK, Lee E, Ko SH, Ahn YB, Kim JW, Yoon KH, Song KH: Activation of AMP-activated protein kinase mediates acute and severe hypoxic injury to pancreatic beta cells. *Biochem Biophys Res Commun* 386(2): 356-62, 2009.

Saldeen J & Welsh N: p38 MAPK inhibits JNK2 and mediates cytokine-activated iNOS induction and apoptosis independently of NF-KB translocation in insulin-producing cells. *Eur Cytokine Netw* 15(1): 47-52, 2004.

Saldeen J, Lee JC, Welsh N: Role of p38 mitogen-activated protein kinase (p38 MAPK) in cytokine-induced rat islet cell apoptosis. *Biochem Pharmacol* 61(12): 1561-9, 2001.

Sato Y, Endo H, Okuyama H Takeda T, Iwahashi H, Imagawa A, Yamagata K, Shimomura I, Inoue M: Cellular hypoxia of pancreatic b-cells due to high levels of oxygen consumption for insulin secretion in vitro. *J Biol Chem* 286(14):12524-32, 2011.

Sato Y, Inoue M, Yoshizawa T, Yamagata K: Moderate hypoxia induces β-cell dysfunction with HIF-1-independent gene expression changes. *PLoS One* 9(12): e114868, 2014.

Sauter NS, Schulthess FT, Galasso R, Castellani LW, Maedler K: The antiinflammatory cytokine interleukin-1 receptor antagonist protects from high-fat diet-induced hyperglycemia. *Endocrinology* 149(5): 2208-18, 2008.

Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, Giallongo A: Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J Biol Chem* 271(51): 32529-37, 1996.

Semenza GL: Hydroxylation of HIF-1: oxygen sensing at the molecular level. *Physiology (Bethesda)* 19: 176-82, 2004.

Semenza GL: Oxygen homeostasis. *Wiley Interdiscip Rev Syst Biol Med* 2 (3): 336-61, 2010.

Shafrir E & Ziv E: Cellular mechanism of nutritionally induced insulin resistance: the desert rodent Psammomys obesus and other animals in which insulin resistance leads to detrimental outcome. *J Basic Clin Physiol Pharmacol* 9(2-4): 347-85, 1998.

Shen J, Chen X, Hendershot L, Prywes R: ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Dev Cell* 3(1): 99-111, 2002.
Shimabukuro M, Zhou YT, Levi M, Unger RH: Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proc Natl Acad Sci U S A* 95(5): 2498-502, 1998.

Schimmer AD: Inhibitor of apoptosis proteins: translating basic knowledge into clinical practice. *Cancer Res* 64(20): 7183-90, 2004.

Simon MN, Azevedo-Martins AK, Amanso AM, Carvalho CRO, Curim R: Persistent activation of Akt or ERK prevents the toxicity induced by saturated and polyunsaturated fatty acids in RINm5F beta-cells. *Toxicol In Vitro* 22(4): 1018-24, 2008.

Skelin M, Rupnik M, Cencic A: Pancreatic beta cell lines and their applications in diabetes mellitus research. *ALTEX* 27(2): 105-13, 2010.

Slee EA, Adrain C, Martin SJ: Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J Biol Chem* 276: 7320-6, 2001.

Sommerweiss D, Gorski T, Richter S, Garten A, Kiess W: Oleate rescues INS-1E β-cells from palmitate-induced apoptosis by preventing activation of the unfolded protein response. *Biochem Biophys Res Commun* 441(4): 770-6, 2013.

Sone H & Kagawa Y: Pancreatic beta cell senescence contributes to the pathogenesis of type 2 diabetes in high-fat diet-induced diabetic mice. *Diabetologia* 48(1): 58-67, 2005.

Song Y, Li X, Li Y, Li N, Shi X, Ding H, Zhang Y, Li X, Liu G, Wang Z: Non-esterified fatty acids activate the ROS-p38-p53/Nrf2 signaling pathway to induce bovine hepatocyte apoptosis in vitro. *Apoptosis* 19(6): 984-97, 2014.

Sperandio S, de Belle I, Bredesen DE: An alternative, non- apoptotic form of programmed cell death. *Proc Natl Acad Sci USA* 97(26): 14376-81, 2000.

Srinivasan K & Ramarao P: Animal models in type 2 diabetes research: an overview. *Indian J Med Res* 125(3): 451-72, 2007.

Stender S & Dyerberg J: Influence of trans fatty acids on health. *Ann Nutr Metab* 48(2): 61-6, 2004.

Stender S, Astrup A, Dyerberg J: Ruminant and industrially produced trans fatty acids: health aspects. *Food Nutr Res* 52, 2008.

Steyn NP, Mann J, Bennett PH, Temple N, Zimmet P, Tuomilehto J, Lindstrom J, Louheranta A: Diet, nutrition and the prevention of type 2 diabetes. *Public Health Nutr* 7(1A): 147-65, 2004.

Stokes RA, Cheng K, Deters N, Lau SM, Hawthorne WJ, O'Connell PJ, Stolp J, Grey S, Loudovaris T, Kay TW, Thomas HE, Gonzalez FJ, Gunton JE: Hypoxia-inducible factor-1α (HIF-1α) potentiates β-cell survival after islet transplantation of human and mouse islets. *Cell Transplant* 22(2): 253-66, 2013.

Surwit RS, Kuhn CM, Cochrane C, McCubbin JA, Feinglos MN: Diet-induced type II diabetes in C57BL/6J mice. *Diabetes* 37(9): 1163-7, 1988.

Šrámek J, Němcová-Fürstová V, Balušíková K, Daniel P, Jelínek M, James RF, Kovář J: p38 MAPK is activated but does not play a key role during apoptosis induction by saturated fatty acid in human pancreatic β-cells. *Int J Mol Sci* 17(2): 159, 2016.

Šrámek J, Němcová-Fürstová V, Kovář J: Kinase signaling in apoptosis induced by saturated fatty acid in pancreatic β-cells. *Int J Mol Sci* 17(9): 1400, 2016.

Šrámek J, Němcová-Fürstová V, Pavlíková N, Kovář J: Effect of saturated stearic acid on MAP kinase and ER stress signaling pathways during apoptosis induction in human pancreatic β-cells is inhibited by unsaturated oleic. *Int J Mol Sci* 2017; 18(11): 2313, 2017.

Tabas I & Ron D: Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat Cell Biol* 13(3): 184-90, 2011.

Takekawa M, Posas, F, Saito H: A human homolog of the yeast Ssk2/Ssk22 MAP kinase kinase kinases, MTK1, mediates stressinduced activation of the p38 and JNK pathways. *EMBO J* 16(16): 4973-82, 1997.

Tanabe K, Liu Y, Hasan SD, Martinez SC, Cras-Meneur C, Welling CM, Bernal-Mizrachi E, Tanizawa Y, Rhodes CJ, Zmuda E, Hai T, Abumrad NA, Permutt MA: Glucose and fatty acids synergize to promote B-cell apoptosis through activation of glycogen synthase kinase 3beta independent of JNK activation. *PLoS One* 6(4): e18146, 2011.

Tanno M, Bassi R, Gorog DA, Saurin AT, Jiang J, Heads RJ, Martin JL, Davis RJ, Flavell RA, Marber MS: Diverse mechanisms of myocardial p38 mitogen-activated protein kinase activation: evidence for MKK-independent activation by a TAB1-associated mechanism contributing to injury during myocardial ischemia. *Circ Res* 93(3): 254-61, 2003.

Taylor CT: Mitochondria and cellular oxygen sensing in the HIF pathway. *Biochem J* 409(1): 19-26, 2008.

Thomas HE, McKenzie MD, Angstetra E, Campbell PD, Kay TW: Beta cell apoptosis in diabetes. *Apoptosis* 14(12): 1389-404, 2009.

Thorn K & Bergsten P: Fatty acid-induced oxidation and triglyceride formation is higher in insulin-producing MIN6 cells exposed to oleate compared to palmitate. *J Cell Biochem* 111(2): 497-507, 2010.

Thorn K, Hovsepyan M, Bergsten P: Reduced levels of SCD1 accentuate palmitate-induced stress in insulin-producing beta-cells. *Lipids Health Dis* 9: 108, 2010.

Thornton CM, Carson DJ, Stewart FJ: Autopsy findings in the Wolcott-Rallison syndrome. *Pediatr Pathol Lab Med* 17(3): 487-96, 1997.

Tian L, Kim HS, Kim H, Jin X, Jung HS, Park KS, Cho KW, Park S, Moon WK: Changes in metabolic markers in insulin-producing β-cells during hypoxia-induced cell death as studied by NMR metabolomics. *J Proteome Res* 12(8): 3738-45, 2013.

Tilly JL, Kowalski KI, Johnson AL, Hsueh AJ: Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. *Endocrinology* 129(5): 2799-801, 1991.

Timmer JC & Salvesen GS: Caspase substrates. *Cell Death Differ* 14(1): 66-72, 2007.

Tinel A & Tschopp J: The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. *Science* 304(5672): 843-6, 2004.

Tiwari M, Lopez-Cruzan M, Morgan WW, Herman B: Loss of caspase-2-dependent apoptosis induces autophagy after mitochondrial oxidative stress in primary cultures of young adult cortical neurons. *J Biol Chem* 286(10): 8493-506, 2011.

Tsujimoto Y & Shimizu S: Another way to die: autophagic programmed cell death. *Cell Death Differ* 12(2): 1528-34, 2005.

Tuei VC, Ha JS, Ha CE: Effects of human serum albumin complexed with free fatty acids on cell viability and insulin secretion in the hamster pancreatic beta-cell line HIT-T15. *Life Sci* 88(17-18): 810-8, 2011.

Tuo Y, Wang D, Li S, Chen C: Long-term exposure of INS-1 rat insulinoma cells to linoleic acid and glucose in vitro affects cell viability and function through mitochondrial-mediated pathways. *Endocrine* 39(2): 128-38, 2011.

Unger RH & Zhou YT: Lipotoxicity of beta-cells in obesity and in other causes of fatty acid spillover. *Diabetes* 50(1): S118-S121, 2001.

Upton JP, Austgen K, Nishino M, Coakley KM, Hagen A, Han D, Papa FR, Oakes SA: Caspase-2 cleavage of BID is a critical apoptotic signal downstream of endoplasmic reticulum stress. *Mol Cell Biol* 28(12): 3943-51, 2008.

Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, Ron D: Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 287(5453): 664-6, 2000.

Vacaresse N, Lajoie-Mazenc I, Auge N, Suc I, Frisach M-F, Salvayre R, Nègre-Salvayre A: Activation of epithelial growth factor receptor pathway by unsaturated fatty acids. *Circ Res* 85(10): 892-9, 1999.

Valencia-Flores M, Orea A, Castano VA, Resendiz M, Rosales M, Rebollar V, Santiago V, Gallegos J, Campos RM, Gonzalez J, Oseguera J, Garcia-Ramos G, Bliwise DL: Prevalence of sleep apnea and electrocardiographic disturbances in morbidly obese patients. *Obes Res* 8(3): 262–69, 2000.

van Loo G, van Gurp M, Depuydt B, Srinivasula SM, Rodriguez I, Alnemri ES, Gevaert K, Vandekerckhove J, Declercq W, Vandenabeele P: The serine protease Omi/HtrA2 is released from mitochondria during apoptosis. Omi interacts with caspase-inhibitor XIAP and induces enhanced caspase activity. *Cell Death Differ* 9(1): 20-6, 2002.

Veluthakal R, Chvyrkova I, Tannous M, McDonald P, Amin R, Hadden T, Thurmond DC, Quon MJ, Kowluru A: Essential role for membrane lipid rafts in interleukin-1beta-induced nitric oxide release from insulin-secreting cells: potential regulation by caveolin-1^{+}. *Diabetes* 54(9): 2576-85, 2005.

Véret J, Coant N, Berdyshev EV, Skobeleva A, Therville N, Bailbé D, Gorshkova I, Natarajan V, Portha B, Le Stunff H: Ceramide synthase 4 and de novo production of ceramides with specific N-acyl chain lengths are involved in glucolipotoxicity-induced apoptosis of INS-1 β-cells. *Biochem J* 438(1): 177-89, 2011.

Walford GA, Moussignac R-L, Scribner AW, Loscalzoand J, Leopold JA: Hypoxia Potentiates Nitric Oxide-mediated Apoptosis in Endothelial Cells via Peroxynitrite-induced Activation of Mitochondria-dependent and -independent Pathways. *J Biol Chem* 279: 4425-32, 2004.

Wang M, Crager M, Pugazhenthi S: Modulation of Apoptosis Pathways by Oxidative Stress and Autophagy in β Cells. *Exp Diabetes Res* 2012: 647914, 2012.

Wang W, Liu Y, Chen Y, Cao C, Xiang Y, Zhang D, Han L, Zhao H, Liu G: Inhibition of Foxo1 mediates protective effects of ghrelin against lipotoxicity in MIN6 pancreatic beta-cells. *Peptides* 31(2): 307-14, 2010.

Watson ML, Macrae K, Marley AE, Hundal HS: Chronic effects of palmitate overload on nutrient-induced insulin secretion and autocrine signalling in pancreatic MIN6 beta cells. *PLoS ONE* 6(10): e25975, 2011.

Wei J, Sun Z, Chen Q, Gu J: Serum deprivation induced apoptosis in macrophage is mediated by autocrine secretion of type I IFNs. *Apoptosis* 11(4): 545-54, 2006.

Welters HJ, Tadayyon M, Scarpello JH, Smith SA, Morgan NG: Mono-unsaturated fatty acids protect against beta-cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure. *FEBS Lett* 560(1-3): 103-108, 2004.

Welters HJ, Diakogiannaki E, Mordue JM, Tadayyon M, Smith SA, Morgan NG: Differential protective effects of palmitoleic acid and cAMP on caspase activation and cell viability in pancreatic beta-cells exposed to palmitate. *Apoptosis* 11(7): 1231-8, 2006.

Wijesekara N, Krishnamurthy M, Bhattacharjee A, Suhail A, Sweeney G, Wheeler MB: Adiponectin-induced ERK and Akt phosphorylation protects against pancreatic beta cell apoptosis and increases insulin gene expression and secretion. *J Biol Chem* 285(44): 33623-31, 2010.

Woehlbier U & Hetz C: Modulating stress responses by the UPRosome: a matter of life and death. *Trends Biochem Sci* 36(6): 329-37, 2011.

Yamamoto K, Yoshida H, Kokame K, Kaufman RJ, Mori K: Differential contributions of ATF6 and XBP1 to the activation of endoplasmic reticulum stress-responsive cis-acting elements ERSE, UPRE and ERSE-II. *J Biochem* 136(3): 343-50, 2004.

Yang G, Yang W, Wu L, Wang R: H_2S, endoplasmic reticulum stress, and apoptosis of insulin-secreting beta cells. *J Biol Chem* 282(22): 16567-76, 2007.

Youl E, Bardy G, Magous R, Cros G, Sejalon F, Virsolvy A, Richard S, Quignard JF, Gross R, Petit P, Bataille D, Oiry C: Quercetin potentiates insulin secretion and protects INS-1 pancreatic β-cells against oxidative damage via the ERK1/2 pathway. *Br J Pharmacol* 161(4): 799-814, 2010.

Yuan H, Zhang X, Huang X, Lu Y, Tang W, Man Y, Wang S, Xi J, Li J: NADPH Oxidase 2-Derived Reactive Oxygen Species Mediate FFAs-Induced Dysfunction and Apoptosis of β-Cells via JNK, p38 MAPK and p53 Pathways. *PLoS ONE* 5(12): e15726, 2010.

Zarubin T & Han J: Activation and signaling of the p38 MAP kinase pathway. *Cell Res* 15(1): 11-18, 2005.

Zehetner J, Danzer C, Collins S, Eckhardt K, Gerber PA, Ballschmieter P, Galvanovskis J, Shimomura K, Ashcroft FM, Thorens B, Rorsman P, Krek W: PVHL is a regulator of glucose metabolism and insulin secretion in pancreatic beta cells. *Genes Dev* 22(22): 3135-46, 2008.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 372(6505): 425-32, 1994.

Zhang S, Han J, Sells MA, Chernoff J, Knaus UG, Ulevitch RJ, Bokoch GM: Rho family GTPases regulate p38 MAP kinase through the downstream mediator Pak1. *J Biol Chem* 270(41): 23934-36, 1995.

Zhang Y, Xu M, Zhang S, Yan L, Yang C, Lu W, Li Y, Cheng H: The role of G protein-coupled receptor 40 in lipoapoptosis in mouse beta-cell line NIT-1. *Mol Cell Endocrinol* 38(6): 651-61, 2007a.

Zhang Y, Padalecki SS, Chaudhuri AR, De WE, Goins BA, Grubbs B, Ikeno Y, Richardson A, Mundy GR, Herman B: Caspase-2 deficiency enhances aging-related traits in mice. *Mech Ageing Dev* 128(2): 213-21, 2007b.

Zheng X, Zheng X, Wang X, Ma Z, Gupta Sunkari V, Botusan I, Takeda T, Björklund A, Inoue M, Catrina SB, Brismar K, Poellinger L, Pereira TS: Acute hypoxia induces apoptosis of pancreatic beta-cell by activation of the unfolded protein response and upregulation of CHOP. *Cell Death Dis* 3: e322, 2012.

Zhou Y, Wang Q, Mark Evers B, Chung DH: Oxidative stress-induced intestinal epithelial cell apoptosis is mediated by p38 MAPK. *Biochem Biophys Res Commun* 350(4): 860-5, 2006.

10. PUBLIKACE NESOUVISEJÍCÍ S TÉMATEM

10.1. Publikace vypracované na základě diplomové práce

Šrámek J, Gvoždík V, Benda P:
Hidden diversity in bent-winged bats (Chiroptera: Miniopteridae) of the Western Palaearctic and adjacent regions: implications for taxonomy.
Zoological Journal of the Linnean Society, 167(1): 165–190, 2013. **IF = 2.658** (2013)

Puechmaille SJ, Allegrini B, Benda P, Gürün K, **Šrámek J**, Ibañez C, Juste J, Bilgin R:
A new species of the Miniopterus schreibersii species complex (Chiroptera: Miniopteridae) from the Maghreb Region, North Africa.
Zootaxa, 3794(1): 108–124, 2014. **IF = 0.906** (2014)

Šrámek J, Benda P:
Sexual and age size variation in the western Palaearctic populations of Miniopterus bats (Chiroptera: Miniopteridae).
Folia Zoologica, 63(3): 216–227, 2014. **IF = 0.724** (2014)

10.2. Publikace vypracováné v rámci doktorského studia

Jelínek M, Balušíková K, Fidlerová J, Němcová-Fürstová V, Kopperová D, **Šrámek J**, Zanardi I, Ojima I, Kovář J:
Caspase-2 is involved in cell death induction by taxanes in breast cancer cells.
Cancer Cell International, 13(1): 42, 2013. **IF = 1.989** (2013)

Jelínek M, Balušíková K, Schmiedlová M, Němcová-Fürstová V, **Šrámek J**, Stančíková J, Zanardi I, Ojima I, Kovář J:
The role of individual caspases in cell death induction by taxanes in breast cancer cells. *Cancer Cell International,* 15(1): 8, 2015. **IF = 2.766** (2014)

Jelínek M, Kábelová A, **Šrámek J**, Ojima I, Kovář J:
Differing mechanisms of death induction by fluorinated taxane SB-T-12854 in breast cancer cells. *Anticancer research*, 37(4): 1581–1590, 2017. **IF = 1.895** (2015)