Charles University in Prague

Faculty of Science

Department of Biochemistry

Mgr. Martina Klevstigová

Myocardial Cell Signaling in Spontaneously Hypertensive Rats with Transgenic and Congenic Expression of Cd36

Ph.D. Thesis

Supervisor: Doc. RNDr. František Novák, CSc.

Doc. RNDr. Olga Nováková, CSc.

Prague, 2013

DECLARATION

I hereby declare that I completed this thesis independently and that I have properly cited all the information sources and literature. This work or a substantial part of it has never been submitted to obtain the same or another academic degree.

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Martina Klevstigová

DECLARATION OF CO-AUTHORS

On the behalf of all co-authors, I hereby declare that Mgr. Martina Klevstigová has substantially contributed (20-90 %) to all 3 articles which represent an integral part of this dissertation. She performed most of the experiments and significantly contributed to the planning of the experiments, interpretation of the results and preparation of the manuscripts.

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Doc. RNDr. Olga Nováková, CSc.

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ABSTRACT

Long-chain fatty acids (LCFA) are the primary energy source in the myocardium and an imbalance in the LCFA and glucose utilization could cause cardiovascular diseases. More than 50% of LCFA uptake by the heart is mediated by the fatty acid translocase CD36 and disruption of its function has been shown to impair cardiovascular functions. The spontaneously hypertensive rat (SHR) harbors a deletion variant of the Cd36 gene that results in reduced LCFA transport into myocytes. Therefore, the main aim of this thesis was to investigate the importance of a functional CD36 to sustain normal physiological functions of the heart. We used SHR and two genetic modified SHR strains, the congenic SHR-4 and the transgenic SHR-Cd36, with fully functional CD36. They differ in the CD36 expression and in the manner how they were derived from the SHR.

CD36 has been proven to play a role in the pathogenesis of insulin resistance. Therefore we analyzed the effect of a functional CD36 on insulin resistance and protein kinase C (PKC) expression, which is known to be involved in the mechanism of insulin resistance, in the heart of SHR-4 and SHR. We showed that the SHR-4 had lower serum free fatty acids (FFA) and triacylglycerols (TAG) concentrations, indicating improved insulin sensitivity. Furthermore, SHR-4 had increased PKC ε expression when compared to the SHR. High sucrose diet (HSD), applied for 14 days, caused the accumulation of heart TAG in the SHR, while increased PKC δ and decreased PKC ε expression was found in the SHR-4. These findings suggest that CD36 in the SHR-4 is associated with reduced insulin resistance, in which PKC δ and ε may play a role.

Fatty acids (FA) are known to be arrhythmogenic. Using the SHR-Cd36, with a wild-type Cd36, we proved that the insertion of Cd36 onto the SHR genome increases the severity and duration of arrhythmias but lowers the myocardial infarct size after coronary occlusion. In addition, we also showed that the higher arrythmogenesis in the SHR-Cd36 is independent of FA uptake but it is rather caused by to increased sensitivity of the β-adrenoceptors (β-AR) signaling pathway documented by higher β-AR density, increased expression of adenylyl cyclase and protein kinase A. Taken together, we proved that the wild-type Cd36 affects the ischemia/reperfusion tolerance in the SHR in β-AR signaling pathway dependent manner.

It can be concluded that the CD36 function plays an important role in various pathophysiological conditions of the heart, including insulin resistance and arrhythmias, which are dependent on PKC isoforms and the β-AR signaling pathway, respectively.

ABSTRAKT

Mastné kyseliny s dlouhým řetězcem (LCFA) jsou primárním zdrojem energie v myokardu. Špatné využití LCFA a glukózy může způsobit kardiovaskulární onemocnění. Více než 50% transportu LCFA do srdce je zprostředkováno translokázou mastných kyselin CD36 a porucha CD36 zhoršuje kardiovaskulární funkce. Spontánně hypertenzní potkan (SHR) má deleční variantu genu pro Cd36, která vede k omezení transportu LCFA do myocytů. Hlavním cílem této práce bylo objasnit význam funkčního CD36 ve fyziologické funkci srdce. Použili jsme dva geneticky modifikované kmeny SHR, kongenní SHR-4 a transgenní SHR-Cd36 s funkčním CD36. Tyto kmeny se liší v expresi CD36 a způsobu, kterým byly odvozeny z kmene SHR.

Bylo prokázáno, že CD36 hraje roli v patogenezi inzulinové rezistence. Z tohoto důvodu jsme v srdcích SHR-4 a SHR analyzovali vliv funkčního CD36 na inzulinovou rezistenci a expresi proteinkinasy C (PKC), která se v mechanismu inzulinové rezistence také uplatňuje. Ukázali jsme, že kmen SHR-4 měl nižší koncentraci volných mastných kyselin (FFA) a triacylglycerolů (TAG) v séru, což naznačuje na zlepšení citlivosti k inzulinu. Dále měl SHR-4 zvýšenou expresi PKC ε ve srovnání s SHR. Vysokosacharózová dieta (HSD), podávaná 14 dnů, způsobila akumulaci TAG v srdcích SHR, zatímco vedla ke zvýšení exprese PKC δ a snížení exprese PKC ε u SHR-4 potkanů. Tyto výsledky naznačují, že přítomnost CD36 u kmene SHR-4 je spojena se sníženou inzulinovou rezistencí, ve které může hrát roli PKC δ a PKC ε.

Je známo, že mastné kyseliny (FA) jsou arytmogenní. Použitím SHR-Cd36 s wild-type CD36 jsme dokázali, že vložení Cd36 na genetické pozadí SHR zvyšuje závažnost a výskyt arytmií ale snižuje velikost infarktu myokardu po koronární okluzi. Dále jsme zjistili, že zvýšená arytmogeneze v SHR-CD36 je nezávislá na transportu FA do srdce, ale je dána spíše větší citlivostí signální dráhy β-adrenergních receptorů (β-AR), což bylo dokumentováno vyšší hustotou β-AR a zvýšenou expresí adenylylcyklasy a proteinkinasy A. Prokázali jsme tak, že wild-type Cd36 ovlivňuje ischemicko/reperfúzní toleranci SHR v závislost na β-AR signální dráze.

Je možné konstatovat, že funkce CD36 hraje důležitou roli v různých patofyziologických podmínkách v srdci, včetně inzulinové rezistence a vzniku arytmií, které jsou závislé na jednotlivých isoformách PKC, respektive na β-AR signální dráze.

LIST OF CONTENTS

LIST OF ABBREVIATIONS

AC	Adenylyl cyclase
AGE	Advanced glycation end product
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AMPKK	AMPK kinase
AR	Area at risk
ATP	Adenosine triphosphate
AWT_D	Diastolic anterior wall thickness
AWT_S	Systolic anterior wall thickness
BN	Brown Norway rat
BSA	Bovine serum albumin
BW	Body weight
C subunit	Catalytic subunit
CaMK	Calcium-calmodulin-dependent protein kinase
CaMKK	CaMK Kinase
cAMP	Cyclic AMP
CPT-I	Carnitine-palmitoyltransferase-I
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
DTT	Dithiothreitol
ECG	Electrocardiogram
ECL	Enhanced chemiluminolescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EPA	Eicosapentaenoic acid
ERK	Extracellulary regulated protein kinases
FA	Fatty acid
FFA	Free fatty acid
FS	Fractional shortening
FSK	Forskolin
G protein	Guanine nucleotide-binding regulatory proteins
GDP	Guaninosine diphosphate
G_i	Inhibitory G protein

GLUT4	Glucose transport protein 4
GPCR	G protein coupled receptor
G_s	Stimulatory G protein
GTP	Guaninosine triphosphate
HMGA1	High-mobility group AT-hook 1
HSD	High sucrose diet
HW	Heart weight
HW/BW	Relative heart weight
i.p.	Intraperitoneal
i.v.	Intravenously
I/R	Ischemia/reperfusion
IRS	Insulin receptor substrate
IS	Infarct size
ISO	Isoproterenol
LAD	Left anterior descending coronary
LCFA	Long-chain fatty acid
LIMP-2	Lysosomal integral membrane protein-2
LKB1	Liver kinase B1
LTCC	L-type Ca^{2+} channel
LV	Left ventricle
LV/BW	Relative left ventricular weight
LVD_D	Diastolic left ventricle diameter
LVD_S	Systolic left ventricle diameter
MAPK	Mitogen activated protein kinase
MI	Myocardial infarction
MUFA	Monounsaturated fatty acid
n	Number of animals
NIH	National Institute of Health
oxLDL	Oxidized low density lipoproteins
PDK	3-Phosphoinositide-dependent protein kinase
PI3K	Phosphatidylinositol-3 kinase
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PKA	Protein kinase A
PKB	Protein kinase B

PKC	Protein kinase C
PKD	Protein kinase D
PLB	Phospholamban
PPAR	Peroxisome proliferator activated receptor
p-PKA	Phosphorylated protein kinase A
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
PVC	Premature ventricular complexes
PWT_D	Diastolic posterior wall thickness
PWT_S	Systolic posterior wall thickness
QTL	Quantitative trait loci
R subunit	Regulatory subunit
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SDS	Sodium dodecyl sulfate
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SFA	Saturated fatty acids
SHR	Spontaneously hypertensive rat
SHR-4	Congenic SHR.BN-IL6/NPY line
SHR-Cd36	Transgenic SHR/O1a-TgN(EF1aCd36)19Ipcv line
SR	Sarcoplasmic reticulum
SRB-1	Scavenger receptor B-1
Src-PTK	Src protein tyrosine kinase
TAG	Triacylglycerol
TnI	Troponin I
TTBS	Tris-buffered saline with Tween 20
TTC	2,3,5-Triphenyltetrazolium chloride
v/v	Volume to volume
VF	Ventricular fibrillation
VFs	Sustained ventricular fibrillations
VT	Ventricular tachycardia
WKY	Wistar-Kyoto rat
β-AR	β-Adrenoceptor

1 INTRODUCTION

1.1 SPONTANOUSLY HYPERTENSIVE RAT

The spontaneously hypertensive rat (SHR) was developed during the 1960s by selective breeding of the normotensive Wistar-Kyoto (WKY) rats with high blood pressure, defined as a systolic blood pressure of over 150 mmHg persisting for more than one month (Okamoto & Aoki, 1963). After 13 generations, the original SHR strain (SHR/Izm) was sent to the National Institute of Health (NIH) Animal Genetic Resource (Bethesda, MD, USA). Thereafter several strains were separated from the SHR/NIH colony and distributed worldwide, among them the SHR/Ola strain. During the 1980's was the SHR/Ola strain brought from the British company OLAC to the Institute of Physiology, Czechoslovak Academy of Science and ever since then has been maintained there by brother-sister mating.

The SHR strain is characterized by hypertension, hypertrophy as well as decreased insulin sensitivity and thus is frequently studied for the understanding of these diseases. Aitman *et al.* (1999) found a deletion in the Cd36 gene, encoding the fatty acid (FA) translocase CD36, in the SHR/NIH strain. CD36 is multifunctional protein; however, one of its main tasks is to transfer long chain fatty acids (LCFA) into the heart. The Cd36 gene, which is located on chromosome 4, became a candidate that could be relevant to the pathogenesis of the metabolic syndrome. Bonen *et al.* (2009) confirmed the findings regarding aberrant Cd36 mRNA expression and insulin resistance in the SHR; although on the contrary to Aitman and colleagues, they found that the CD36 protein was expressed in reduced levels in the SHR/NIH strain. These studies led to the conclusion that SHR/NIH strain has reduced levels of the CD36 protein leading to reduced FA uptake. On the other hand, the original Japanese SHR/Izm strain, which has been maintained in Japan since 1966, has no mutation in the Cd36 gene (Gotoda *et al.*, 1999), indicating that the observed discrepancy in the CD36 functioning might result from *de novo* mutation during breeding in NIH. However, in this work we always used SHR with Cd36 mutation.

Genetic studies have become a useful tool in the clarification of different molecular mechanisms and during the last decade different genetically modified strains have been derived from the SHR. The congenic SHR-4 and transgenic SHR-Cd36 were prepared for

studying the role of the FA translocase CD36 in hypertension, insulin resistance and ischemic tolerance.

1.1.1 SHR and hypertension

The SHR is the most widely studied animal model of human essential hypertension (Pravenec & Kurtz, 2010). The rise in blood pressure begins around 5-6 weeks of age and the systolic pressure may reach values between 180 and 200 mmHg in the adulthood. In addition, hypertrophy of the heart and vessels parallels precedes hypertension development in SHR (Sen *et al.*, 1974; Mulvany & Nyborg, 1980). By the age of 18-24 months, the SHR develops physical signs of dilated heart failure (e.g. resting tachycardia, tachypnea) (Bing *et al.*, 1995) and thus is as well frequently used to study heart failure. Number of studies tried to identify genes that could influence the hypertension in the SHR (Aitman *et al.*, 1997; Aitman *et al.*, 1999; Pravenec *et al.*, 2001; Pravenec *et al.*, 2008a; Pravenec *et al.*, 2008b). However, using congenic and transgenic strains, the only gene confirmed to affect the blood pressure in the SHR was the Cd36 gene (Pravenec *et al.*, 2008a).

Under appropriate dietary conditions the SHR exhibits, together with hypertension, multiple metabolic disorders similar to the human metabolic syndrome (Aitman *et al.*, 1997; Pravenec *et al.*, 1999; Pravenec *et al.*, 2001). Studies have proved that the alterations in lipid and/or carbohydrate metabolism can influence the regulation of blood pressure and the development of hypertension (Reaven & Chang, 1991).

1.1.2 SHR and the metabolic syndrome

The metabolic syndrome is a common cluster of risk factors for coronary heart disease and type 2 diabetes mellitus. Several professional societies have developed definitions of the metabolic syndrome that includes somewhat different components and cut-off points. Nevertheless, the mutual components of the metabolic syndrome are obesity, elevated blood pressure, dyslipidemia and mainly insulin resistance (Grundy *et al.*, 2005; Zimmet *et al.*, 2005).

Insulin resistance, which is broadly defined as the reduction in insulin ability to stimulate peripheral tissue glucose uptake (Reaven, 1993), can be induced in the SHR by high-carbohydrate diet and is accompanied by dyslipidemia and hyperinsulinemia

(Pravenec *et al.*, 1999). Therefore, SHR is frequently used to study different proteins (G protein-coupled receptor kinase 2, CD36, C-reactive protein) supposed to be involved in insulin resistance of insulin-sensitive tissues, i.e. adipose tissue and skeletal muscle. Although number of studies has successfully improved the insulin resistance in the SHR through protein stimulation, inhibition and/or genetic modification (Rizzoni *et al.*, 2008; Cipolletta *et al.*, 2009; Pravenec *et al.*, 2011), the pathway leading to insulin resistance in SHR still remains unsolved. The protein kinase C (PKC) isoforms could be a plausible component in the mechanism of insulin resistance in the SHR. PKC can inhibit several steps of the insulin signaling cascade, including contraction-induced recruitment of the glucose transporter protein 4 (GLUT4) and CD36 from intracellular stores to sarcolemma of cardiac myocytes (Coort *et al.*, 2007; Schmitz-Peiffer *et al.*, 2007).

1.1.3 SHR and the heart

Cardiac hypertrophy is an adaptive enlargement of the myocardium, compensatory for an increased workload caused by hypertension, various stress or injury; however, the prolongation of this process leads to congestive heart failure, arrhythmias and sudden death (Akazawa & Komuro, 2003). It is evident that SHR has an increased heart mass in comparison to control rats (Hajri *et al.*, 2001; Pravenec *et al.*, 2011) and the mutation of the FA translocase Cd36 could contribute to this phenomenon. The SHR heart, due to nonfunctional CD36, is not receiving its main energy substrate, LCFA, and thus is put into a stress situation. Nevertheless, study with medium chain FA, energy substrate that do not require CD36 transport to enter cardiomyocytes, has shown to abolish hypertrophy in SHR (Hajri *et al.*, 2001). Furthermore, it has as well been established that the hypertrophy in SHR is associated with decreased FA transport and metabolism (Purushothaman *et al.*, 2011). In addition to hypertrophy, when compared to control strains, the SHR exhibits differences in a number of important cardiac phenotypes including the impaired ischemic tolerance (Kolar & Parratt, 1997; Dai *et al.*, 2009; Ravingerova *et al.*, 2011) and increased susceptibility to ventricular arrhythmias (Leenen & Yuan, 2001; Ravingerova *et al.*, 2011). One important pathway involved in both hypertrophy and ischemic tolerance is the β-adrenoceptor (β-AR) mediated pathway (Tong *et al.*, 2005; Tang *et al.*, 2011). Nevertheless, it has been proven that transient activation of β-AR can elicit protection from ischemia/reperfusion (I/R) injury in heart (Tong *et al.*, 2005). The SHR has altered β-AR

sensitivity in comparison with control strains, thus administration β-AR antagonists failed to suppress the rise of arterial pressure and hypertrophy development in the SHR (Pfeffer *et al.*, 1977).

1.2 CD36 CONGENIC SPONTANEOUSLY HYPERTENSIVE RATS

The congenic SHR.BN-IL6/NPY line (hereafter referred as SHR-4) was developed to confirm the involvement of Cd36 as a candidate gene in the pathogenesis of the metabolic syndrome (Pravenec *et al.*, 1999). The SHR-4 congenic strain was derived by transferring a segment of chromosome 4 (Fig 1) from the inbred normotensive Brown Norway (BN) strain into the genetic background of the SHR/O1a strain.

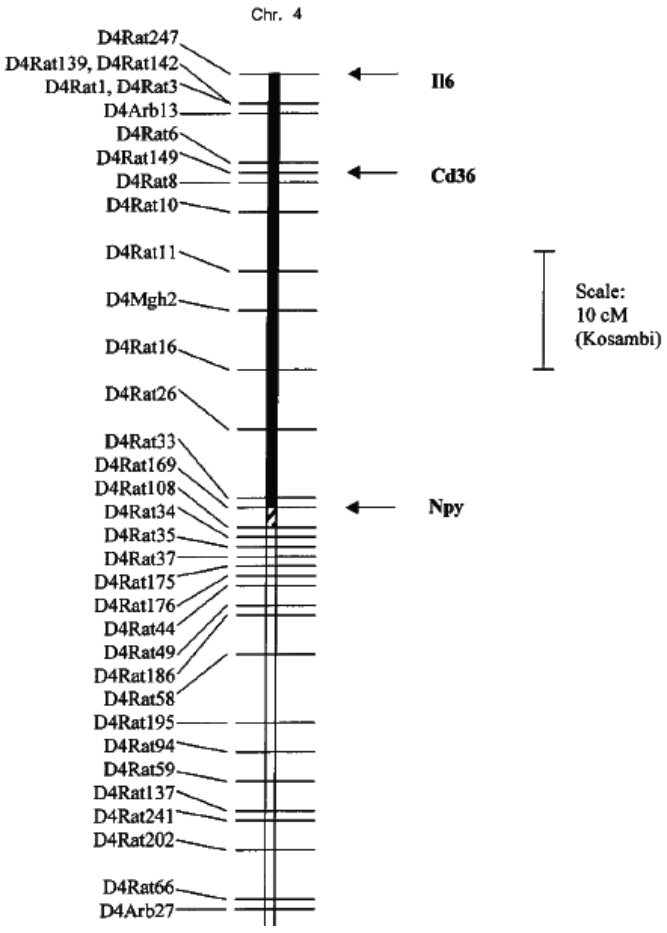

Fig 1: Linking map showing the transferred segment of chromosome 4 in the spontaneously hypertensive rat congenic strain (SHR-4). The filled bar denotes the chromosome region from the Brown Norway (BN) strain that has been fixed in the homozygous state on the spontaneously hypertensive rat (SHR) background. The cross-hatched bar denotes region in which some residual heterozygosity may exist within the congenic strain. The open region denotes the flanking segment of SHR chromosome. The position of Cd36 is based on radiation hybrid mapping. Adapted and modified from Pravenec et al. (1999).

The SHR-4 congenic strain is by 99% genetically identical to the SHR progenitor strain. It was demonstrated that the SHR-4 exhibits lower blood pressure than the progenitor SHR

strain, and lower concentrations of metabolic cardiovascular risk factors (serum insulin, triacylglycerol (TAG) and FA concentrations) after high carbohydrate diet feeding (Pravenec *et al.*, 1999). These metabolic changes were also accompanied by increased glucose tolerance when compared to the progenitor SHR strain (Pravenec *et al.*, 1999). Hajri *et al.* (2001) have proven an increase in FA uptake in both heart and adipose tissue in the congenic strain when compared to progenitor SHR controls.

1.3 CD36 TRANSGENIC SPONTANEOUSLY HYPERTENSIVE RATS

The CD36 transgenic SHR/O1a-TgN(EF1aCd36)19Ipcv line (hereafter referred as SHR-Cd36) was derived to prove that defective Cd36 in SHR is a quantitative trait loci (QTL) for insulin resistance and disordered FA metabolism (Pravenec *et al.*, 2001). The SHR-Cd36 strain was prepared by microinjecting SHR/O1a zygotes with wild-type Cd36 cDNA isolated from fat tissue of the WKY rat (Pravenec *et al.*, 2001). While the congenic SHR-4 strain is 99% genetically identical, the SHR-Cd36 is, except the wild-type Cd36 gene, 100% identical to the SHR progenitor strain. The transgenic strain harbors a single copy of the transgene and has improved glucose tolerance and serum FA levels in comparison to the progenitor SHR strain. Although the SHR-Cd36 transgenic strain has lower Cd36 mRNA expression and membrane associated CD36 protein level when compared to WKY, the Cd36 transgene rescue corrected the SHR muscle defect in insulin action (Pravenec *et al.*, 2001). Similarly to the congenic SHR-4 strain, the SHR-Cd36 has, although not to the same extent, lower blood pressure when compared to the SHR progenitor strain. On the other hand, the transgenic SHR-Cd36 strain has greater relative protein expression of CD36 when compared to the congenic SHR-4 strain and the normotensive BN strain, CD36 was reported to be a factor involved in the blood pressure regulation (Pravenec *et al.*, 2008a).

1.4 PROTEIN KINASE C

PKC is a family of serine/threonine kinases. The PKCs are major mediators of signal transduction pathways and have been shown to regulate sets of biological functions as diverse as cell growth, differentiation, apoptosis, transformation and tumorigenicity. The PKC family is composed of a number of individual isoforms which belong to three distinct classes, classical (α, βI, βII, γ), novel (δ, ε, θ and η) and atypical (ζ, ι) based upon their structurally distinct N-terminal regulatory domain (Sampson & Cooper, 2006; Duquesnes *et al.*, 2011). According to the difference in the binding capability of their regulatory domain, the PKC classes are regulated by different second messengers. The classical PKCs are regulated by diacylglycerols (DAG), calcium, phosphatildylserine (PS) and phosphatidylinositol-4,5-bisphosphate (PIP_2). Novel PKCs are activated by DAG, PS and PIP_2 and the atypical PKCs, which are insensitive to both DAG and calcium, are regulated by PS (Newton, 2010). The PKC isoforms are ubiquitously expressed in tissues and organs and the most abundant isoforms in the heart are PKC α, δ, ε and ζ (Rybin & Steinberg, 1994).

The novel PKCs isoforms, δ and ε, have both been implicated in a variety of cardiac pathological conditions, such as cardiac hypertrophy, heart failure and I/R injury as well as in cardioprotection (Duquesnes *et al.*, 2011). The first indication that the PKCs are involved in cardioprotection came in the 1990's when a broad non-specific inhibition of PKC isoforms lead to abolishment of the cardioprotective effect of ischemic preconditioning (Ytrehus *et al.*, 1994). The development of specific PKC isoform inhibitors and agonists has permitted the investigation of the role of specific PKC isoforms in I/R injury. *In vivo* studies have shown that the application of PKC ε specific agonist prior ischemia or PKC δ specific inhibitor at reperfusion resulted in protection of the heart (Chen *et al.*, 2001; Inagaki *et al.*, 2003). The exact mechanism leading to cardioprotection is still not known. Nevertheless, the use of specific compounds and transgenic animal models allows investigators to get closer to the elucidation of this pathway. It is of interest that, although PKC ε and δ have opposing effect in cardioprotection, the activation of either isoform leads to heart hypertrophy in numerously different animal models (Chen *et al.*, 2001; Duquesnes *et al.*, 2011).

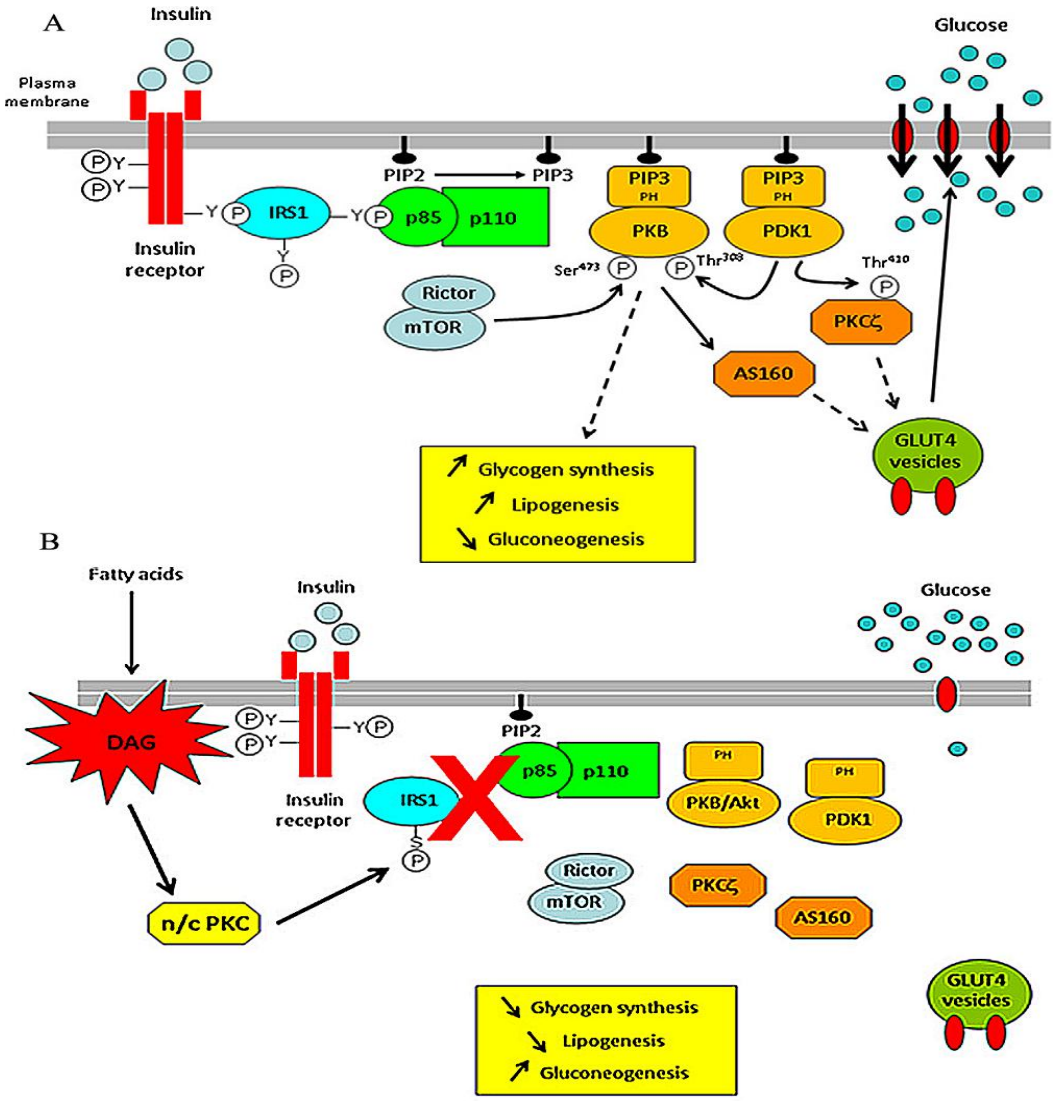

Fig 2: Insulin signaling pathway. (A) Insulin activates the insulin receptor kinase, leading to the activation of insulin receptor substrates (IRSs), phosphatidylinositol-3 kinase and protein kinase B/Akt (PKB/Akt). Activated PKB/Akt promotes GLUT4 translocation to the plasma membrane and glucose uptake in adipocytes and skeletal muscle, as well as glycogen synthesis in skeletal muscle and liver. In liver, insulin promotes lipogenesis and inhibits gluconeogenesis. (B) Implication of protein kinase C (PKC) isoforms in the development of fatty acid induced insulin resistance. Insulin-resistance results from excess accumulation of fatty acids in ectopic tissues, leading to production of lipid metabolites like diacylglycerols (DAG). High levels of DAG in insulin sensitive tissue impair insulin signaling at the level of IRSs. DAG is thought to act through both conventional and novel PKCs that induce the phosphorylation of IRSs on serine residues, thus reducing signaling to downstream effectors. Adapted and modified from Turban & Hajduch (2011)

It has been shown that PKC ε and PKC δ influence the key steps in glucose and energy metabolism in the heart. Using transgenic strains Mayr *et al.* (2004) proved that PKC ε

activation or PKC δ ablation caused changes in the expression of several enzymes related to glucose and energy metabolism, including pyruvate kinase and lactate dehydrogenase, which were downregulated in these models. Furthermore, the enzymes related to lipid metabolism were increased in hearts of PKC δ knockout mice, indicating a metabolic shift from glycolysis toward increased lipid metabolism (Mayr *et al.,* 2004). Multiple studies have indicated that PKCs have also a role in the development of diabetic cardiomyocyte dysfunction (Koya & King, 1998). Elevated PKC activity has been found in insulin-resistant cardiomyocytes and PKC inhibition normalized the impaired myocytes function (Davidoff *et al.,* 2004). Both PKC ε and δ have transduction as well as modulating effects on insulin action. They are both activated by insulin and both are to be negative regulators in the insulin signaling pathway. Upon insulin activation, PKC ε plays a role in the translocation of the glucose transporters from its vesicle storage compartments to the plasma membrane (Niu *et al.,* 2011), while the possible role for PKC δ after insulin activation is still to be resolved. Activation of PKC ε by FA inhibits the gene transcription of the insulin receptor and causes insulin resistance, whereas activated PKC δ phosphorylates the insulin receptor (Fig 2) and prevents further interaction between the receptor and its substrates (Bhattacharya *et al.,* 2007). Furthermore, deletion of PKC ε or δ improves glucose tolerance and reduces lipid accumulation, two important factors in insulin resistance, in mice fed a high-fat diet (Frangioudakis *et al.,* 2009).

1.5 β-ADRENOCEPTOR SIGNALING PATHWAY

G protein coupled receptors (GPCRs) are a conserved family of seven transmembrane spanning receptors that represent the majority of receptors in the genome. β-ARs, which are members of the GPCRs, mediate a variety of functions, including positive inotropic and chronotropic responses of the heart (El-Armouche & Eschenhagen, 2009). They are coupled to the heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins), which regulate the enzyme activity of adenylyl cyclase (AC) (Rockman *et al.,* 2002). AC catalyzes the conversion of cellular ATP into cyclic AMP (cAMP), a key second messenger molecule, which activates protein kinase A (PKA) by binding to the regulatory peptide and altering its conformation (Xiao *et al.,* 2006). Activated PKA then enhance the

cardiac contraction through phosphorylation of plasmalemma and sarcomeric ion channels and myofibril contractile proteins (Xiang & Kobilka, 2003).

Three subtypes of β-ARs have been found in the adult heart. In the healthy mammalian heart β_1-ARs account for the majority (70-80%) of total β-ARs, while β_2-ARs account for approximately 20-30% and β_3-ARs have a minimal contribution. The β_1-ARs and β_2-ARs are highly homologous both structurally and functionally and they share 52% identity overall and 76% identity in the transmembrane domains. Despite the similarity, β_1-ARs and β_2-ARs signal through distinct pathways (Xiang & Kobilka, 2003). Whereas stimulated β_1-ARs couple only to the stimulatory G protein (G_s) and play a dominant role in increasing contractility in cardiac myocytes and apoptosis, activated β_2-ARs couples both to the G_s and the inhibitory G protein (G_i). Thereby they induce not only a positive inotropic effect but also antiapoptosis (Brodde *et al.*, 2006) (Fig 3).

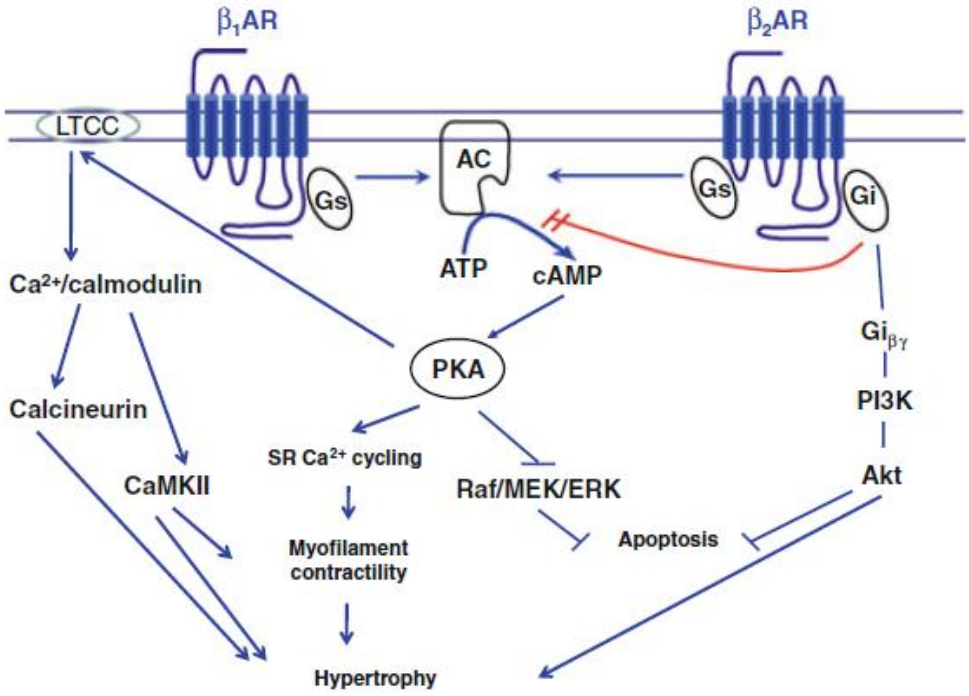

Fig 3: Schematic representation of β-adrenoceptor (β-AR) signaling pathway in cardiomyocytes. Upon β-AR stimulation, the Gsα subunit bound to adenylyl cyclase (AC) produces cAMP which activate target molecule, protein kinase A (PKA). PKA regulates Ca^{2+} homeostasis through sarcoplasmic reticulum (SR) and L-type Ca^{2+} channel (LTCC), increasing thus myocardial contractility. Under the conditions of excessive sympathetic stimulation, PKA induced Ca^{2+} elevation leads to hypertrophy. On the other hand, activated PKA inhibits Raf, which results in the activation of the MEK/ERK signaling, and thus suppression of apoptosis. Adapted and modified from Ho et al. (2010)

The importance of β-ARs, especially β_1-ARs, in the cardiac performance is known for decades, while the β_3-AR was not discovered until 1989. The β_3-ARs differ from β_1-ARs and β_2-ARs by their molecular structure and pharmacological profile e.g. they require higher catecholamine concentration for their activation. Unlike β_1-ARs and β_2-ARs, the stimulated β_3-ARs couple only to the G_i and induce a negative inotropic effect (Gauthier *et al.*, 2007). The observations in knockout mice proved that contraction can be induced in myocytes in the absence of β_2-AR (Soto *et al.*, 2009). These data indicate that the contraction response is primarily due to β_1-AR stimulation. Furthermore, β_1-ARs antagonists are frequently used in the treatment and prevention of arrhythmias (Workman, 2010). However, β_2-ARs have been shown to induce contractility in pathological cases such as after myocardial ischemia and during heart failure. Stimulation of β-ARs activates the G_s protein by promoting the exchange of GDP for GTP. The dissociated GTP bound $G_s\alpha$ subunit then binds to and activates AC. AC is a transmembrane protein capable of converting ATP to cAMP upon stimulation (Ho *et al.*, 2010). cAMP, an intracellular second messenger, is involved in a multitude of physiological functions e.g. contractility response. Up to date, at least nine mammalian isoforms of AC, AC1-AC9, have been identified, and each isoform has a distinct tissue distribution, biological as well as pharmacological properties. AC5 and AC6 are the major AC isoforms in the heart (Defer *et al.*, 2000). Although AC5 and AC6 share a very high amino acid sequence identity (65%) and share several regulatory properties, they exert opposite effects in disease states, particularly in the cardiovascular system (Beazely & Watts, 2006). Using transgenic mice, it was shown that deletion of AC5 protects against the development of apoptosis, impairment of heart function and heart failure (Okumura *et al.*, 2007), while AC6 overexpression abrogates hypertrophy and improves heart function in cardiomyopathy (Roth *et al.*, 2002).

PKA is key regulatory enzyme in the heart that is involved in the contraction coupling. In the inactive state, PKA exists as a tetramer, consisting of two regulatory (R) and two catalytic (C) subunits. Binding of cAMP to the R subunits results in dissociation of the C subunits and activation of the enzyme (Skalhegg & Tasken, 2000). The free C subunits can then affect a range of diverse cellular events by phosphorylating numerous cytoplasmic and nuclear substrates (Cheng *et al.*, 2008). Up to date four R subunits (RIα, RIβ, RIIα and RIIβ) and three C subunits (α, β and γ) have been cloned. The R subunits exhibit different

cAMP binding affinities and are differently expressed in various cells and tissue. Their ability to form both homo- and heterodimers generates a large number of combinations, which further contribute to diversity and presumably specificity in the cAMP signal pathway (Di Benedetto *et al.*, 2008). One of the PKAs main functions in the heart is to regulate cardiac contractility. Upon the activation PKA phosphorylates and activates several proteins that directly or indirectly control sarcomere contraction and potential action duration. In particularly, phosphorylation of L-type Ca^{2+} channel (LTCC) and ryanodine receptor 2 (RyR) enhances Ca^{2+} mobilization from intracellular stores, and favors cardiomyocyte contraction, whereas phosphorylation of phospholamban (PLB), a negative regulator of the activity of the sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2) pump, promotes SERCA2 activation and reuptake of Ca^{2+} into the sarcoplasmic reticulum (SR), which favors cardiomyocyte relaxation. Moreover, PKA also influences the dynamics of cardiomyocyte contraction by phosphorylating the sarcomeric protein troponin I (TnI) and myosin binding protein C (Tasken & Aandahl, 2004). PKA plays an important role in the pathogenesis of the heart failure. Mice overexpressing PKA showed RyR 2 and PLB hyperphosphorylation that was accompanied by dilated cardiomyopathy, arrhythmias and sudden death (Antos *et al.*, 2001). Furthermore, during prolonged β-ARs stimulation, e.g. during hypertrophy, PKA phosphorylates and desensitizes the β-AR, which could lead to heart failure (Edwards *et al.*, 2012).

1.6 FATTY ACID TRANSLOCASE CD36

Fatty acid translocase/CD36 (sometimes referred as FAT/CD36) was first described as a transmembrane glycoprotein homologous to the human scavenger receptor CD36 (cluster of differentiation 36) and is expressed in a wide range of cells and tissues, including cardiac and skeletal muscle, monocytes and macrophages. CD36 mediates a broad list of functions, it is prominent in the transport of LCFA into the cells, cardiovascular function and disease, muscle function and metabolism (Febbraio & Silverstein, 2007).

1.6.1 Structure

CD36 is the defined member of a gene family that includes two other members, lysosomal integral membrane protein-2 (LIMP-2) and scavenger receptor B-1 (SRB-1) (Martin *et al.*, 2011). The apparent molecular mass of human and rodent CD36 is 88 kDa by reason of posttranslational glycosylation while the non-glycosylated protein has a mass of 55 kDa. CD36 contains 471 amino-acid residues and has a predicted receptor like structure (Fig 4).

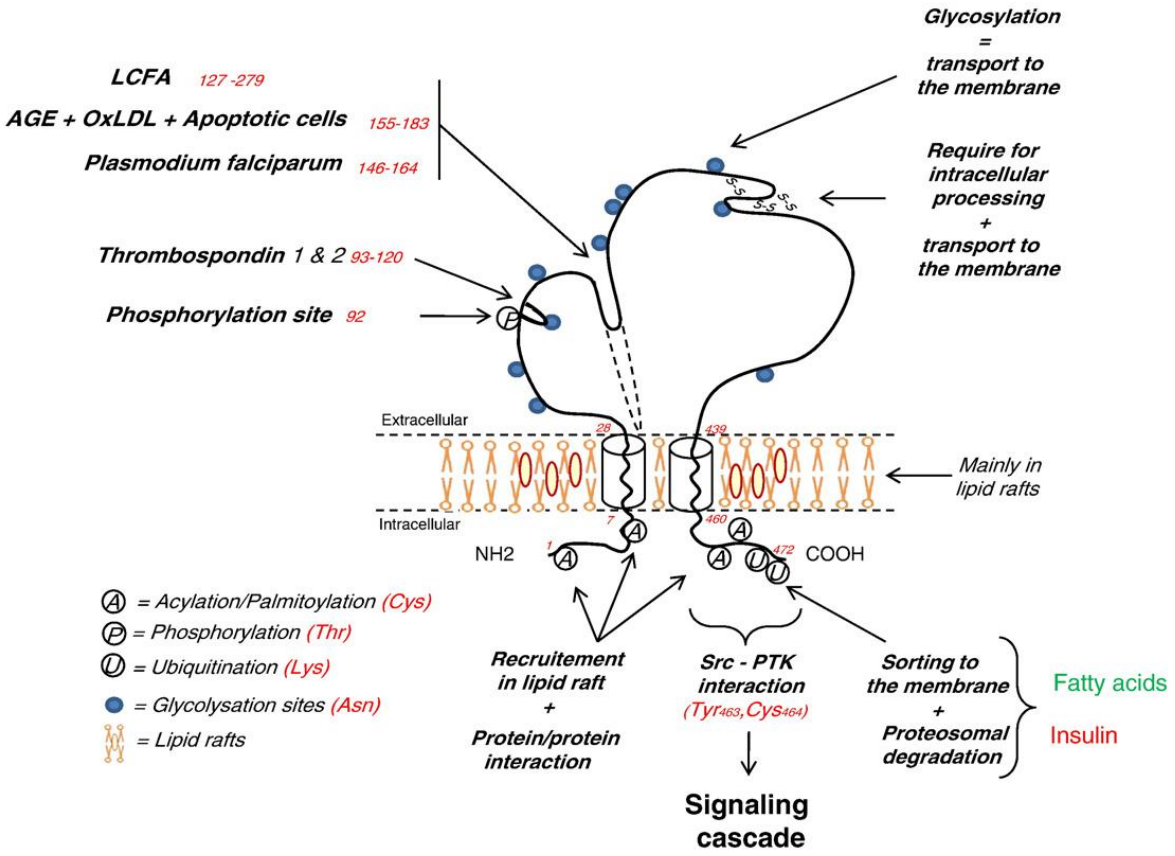

Fig 4: Main structural features of human CD36. Advanced glycation end products (AGE); long-chain fatty acids (LCFA); oxidized low density lipoproteins (OxLDL); Src protein tyrosine kinases (Src-PTK). Adapted from Martin et al. (2011)

CD36 has two transmembrane domains and a hairpin configuration with an extracellular domain containing at least two binding sites. The first one is a thrombospondin-binding site and the second constitutes a large hydrophobic pocket containing different domains able to bind specific molecules responsible for the recognition of LCFA, erythrocytes infected with *Plasmodium falciparum*, oxidized low

density lipoproteins (oxLDL), advanced glycation end products (AGE) or apoptotic cells (Martin *et al.*, 2011). Different posttranslational modifications contribute to the function of CD36. The three disulfide bridges on the extracellular domain are essential for the intracellular processing of the protein and its correct targeting towards the plasma membrane (Martin *et al.*, 2011). Glycosylation of the asparagine sites have an important role in the trafficking of CD36 to the membrane, whereas a minimum of three glycosylated asparagines (247, 321, and 417) is required for the ability to traffic (Hoosdally *et al.*, 2009). Furthermore, acylation and/or palmitoylation of the N- and C-terminal cysteine residues contribute to the recruitment of CD36 in the lipid rafts (Martin *et al.*, 2011). The ubiquitination of lysine 469 and 472 on the C-terminal tail have been proven to be a mechanism by which CD36 is acutely regulated by insulin and FA. Whereas insulin increased the ubiquitination, which was followed by increased FA uptake and its incorporation into TAG, FA had the opposite effects, i.e. decreased the ubiquitination and FA uptake (Smith *et al.*, 2008). Furthermore, the ubiquitination is also vital for the degradation of the protein as inhibition of proteasomes led to the accumulation of ubiquitinated CD36 (Smith *et al.*, 2008).

1.6.2 Function

CD36 has a diverse expression pattern and multiple cellular functions (Fig 5). In microvascular endothelial cells, CD36 functions as a receptor for thrombospondin 1 and as an endogenous regulator of angiogenesis and therefore plays a role in tumor growth, inflammation, wound healing and other pathological processes requiring neovascularization. CD36 inhibits growth factor induced proangiogenic signals that mediate endothelial cell proliferation, migration, and tube formation and instead generating anti-angiogenic signals that lead to apoptosis (Silverstein & Febbraio, 2009).

As a scavenger receptor – that is, a pattern recognition receptor – CD36 recognizes lipid and lipoprotein components of bacterial cell walls and thereby triggers a reaction that results in opsonin-dependent pathogen internalization. CD36 also recognizes endogenously derived ligands, including apoptotic cells, oxidatively modified lipoproteins, glycated proteins and amyloid forming peptides (Silverstein *et al.*, 2010).

Recognition and internalization of oxLDL by macrophage CD36 promotes formation of lipid-laden foam cells and atherosclerotic plaque, triggers different proinflammatory

reactions (release of cytokines, production of reactive oxygen species (ROS)) and inhibits macrophage migration (Silverstein & Febbraio, 2009). Furthermore, the interaction between CD36 and oxLDL on blood platelets could represent a link between oxidative stress, hyperlipidemia, inflammation and pathological thrombosis (Ashraf & Gupta, 2011). On dendritic cells of the immune system, CD36 provides a mechanism for cross-presentation of antigens to cytotoxic T-cells by mediation uptake of apoptotic cells (Febbraio & Silverstein, 2007).

Studies on CD36 knockout mice have shown that CD36 is an important component in satiety induced by dietary fat (Schwartz *et al.*, 2008). The consequence of CD36 binding and signaling is apparently both ligand and cell type dependent. Furthermore, the signaling cascade initiated by CD36 often appears to include the activation of src kinase that leads to MAP kinase (MAPK) activation (Febbraio & Silverstein, 2007).

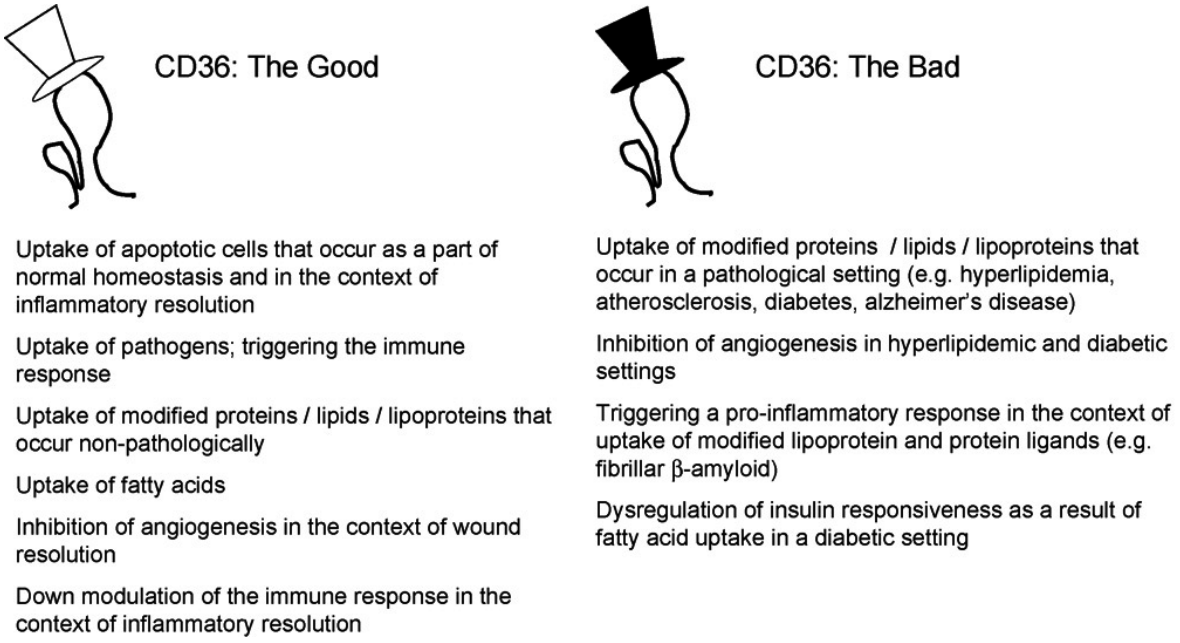

Fig 5: Functions of CD36 are homeostatic and contribute to normal cellular responses (CD36: The Good). In certain contexts, functions of CD36 contribute to pathological states (CD36: The Bad). Adapted from Febbraio and Silverstein (2007)

The CD36 function as LCFA transporter was discovered by Abumrad and her colleagues when they by means of reactive sulfo-N-succinimidyl esters of LCFA inhibited the FA uptake in adipocytes (Harmon & Abumrad, 1993). The important role of CD36 in FA uptake was proven by studies on CD36 knockout mice, where the FA uptake was

reduced by 80% in the heart, 75% in skeletal muscle and 70% in adipose tissue (Coburn *et al.*, 2000). Recent studies showed that CD36 is also located on the outer mitochondrial membrane and is important in the LCFA transport across the mitochondrial membrane and FA oxidation (Smith *et al.*, 2011). The theory is that CD36 works in conjunction with carnitine-palmitoyltransferase-I (CPT-I), as there is a high correlation between the rate of FA oxidation and the combined effects of mitochondrial CPT-I activity and CD36 expression in isolated mitochondria (Bezaire *et al.*, 2006).

There is evidence suggesting that CD36 works in coordinated manner in the LCFA cell uptake. Number of studies have described an interaction between CD36 and the plasma membrane FA binding protein for the uptake of LCFA. In some cells, expression of caveolin-1 has been shown to be necessary for plasma membrane targeting of CD36 and thus for LCFA transport across the membrane. CD36 may also work together with FA transport protein 6 as they colocalize in the heart (Glatz *et al.*, 2010).

60-90% of the heart energy is provided by LCFA β-oxidation (Vandervusse *et al.*, 1992) and since the intramuscular storages are a limited source, the heart must rely on the continuously supply of exogenous LCFA. It is known that up to 80% of the heart LCFA uptake is protein mediated (Dirkx *et al.*, 2011) and this protein mediated cardiac LCFA uptake is predominantly dependent on CD36. Nevertheless, plasma membrane FA binding protein and FA transport protein 1 and 6 also contribute to the FA uptake in the heart although in a smaller extend.

1.6.3 Regulation

The regulation of CD36 can be influenced by a number of actions (e.g. contraction) and/or signaling components (e.g. insulin). Nevertheless, CD36 is under the transcriptional control of the nuclear peroxisome proliferator activated receptor (PPAR) family in a tissue-specific manner. Depending on the presence of the three PPAR subtypes, α, β/δ and γ, cells will respond to specific PPAR agonist by a transcriptional regulation of the CD36 expression (Glatz *et al.*, 2010). Another level of CD36 regulation is through the translocation from intracellular storages to the plasma membrane. Significant intracellular pools of CD36 have been found in monocytes/macrophages and muscle (Febbraio & Silverstein, 2007). Insulin and myocardial contraction are the main physiological stimuli able to induce FA uptake through the translocation of CD36 from its storage to the

membrane (Fig 6). Whereas the insulin signaling cascade starts from the extracellular leaflet of sarcolemma, the origin of the contraction signaling cascade is intracellular. It has been shown that the signaling pathway leading to relocation of CD36 to the sarcolemma is similar to the pathway that leads to the translocation of the glucose transport protein GLUT4 (Glatz *et al.*, 2010).

From all stimulus-induced transporter translocation processes, insulin-induced GLUT4 translocation is the most studied. Several of the kinases involved in this relocation to the sarcolemma seem to play a similar role in the insulin-induced CD36 translocation (Fig 6A) (Steinbusch *et al.*, 2011). The binding of insulin to the α-subunit of the insulin receptor induces a conformational change throughout the whole protein, resulting in the autophosphorylation of the β-subunit, thereby providing docking site for the insulin receptor substrate 1 (IRS-1). In addition, IRS-1 recruits members of the phosphatidylinositol-3 kinase (PI3K) family to the plasma membrane and activates them. Activated PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP_2) into phosphatidylinositol-3,4,5-triphosphate (PIP_3). The generation of PIP_3 at the plasma membrane directly drives the activation of a number of different protein kinases with lipid binding pleckstrin homology domains. Akt/protein kinase B (PKB) isoform and the PKC λ/ζ are two kinases that play an essential role in insulin-induced CD36 translocation in muscle (Glatz *et al.*, 2010).

Increased heart contractile activity can result in a swift rise in the concentration of a number of second messengers, such as AMP, cAMP, Ca^{2+} and ROS. Together these second messengers activate a complex network that involves AMP-activated protein kinase (AMPK), PKA, PKCs, protein kinase D (PKD), calcium-calmodulin-dependent protein kinases (CaMK), and the extracellulary regulated protein kinases (ERK) 1 and 2 (Fig 6B). AMPK is known to have a variety of metabolic actions and to play a crucial role in the translocation of CD36 (Luiken *et al.*, 2004). Cardiac work increases the AMP/ATP ratio and, subsequently, AMPK activity. Binding of AMP to the regulator domains of AMPK leads to conformational changes and makes the protein accessible for upstream AMPK kinases (AMPKK), which phosphorylate AMPK and consequently activate it. The CaMK kinase (CaMKK) and the tumor suppressor protein kinase, liver kinase B1 (LKB1), are suggested kinases with AMPKK activity (Hawley *et al.*, 2005; Woods *et al.*, 2005; Habets *et al.*, 2009). Several kinases have been reported to act downstream of AMPK in the CD36

translocation, most notably ERK1/2 and atypical PKCs (Chen *et al.*, 2002). The insulin; or contraction-induced signaling cascades initiate a sequence of trafficking events that result in the translocation of CD36. Cellular protein traffic generally involves three major steps. First, vesicle fission that is dependent on destabilizing proteins, coat proteins and Rab GTPases. Secondly, subcellular vesicle transport regulated by Rab GTPases. Thirdly, vesicle fusion that is dependent on the formation of a SNARE complex and is modulated by Rab GTPases (Glatz *et al.*, 2010).

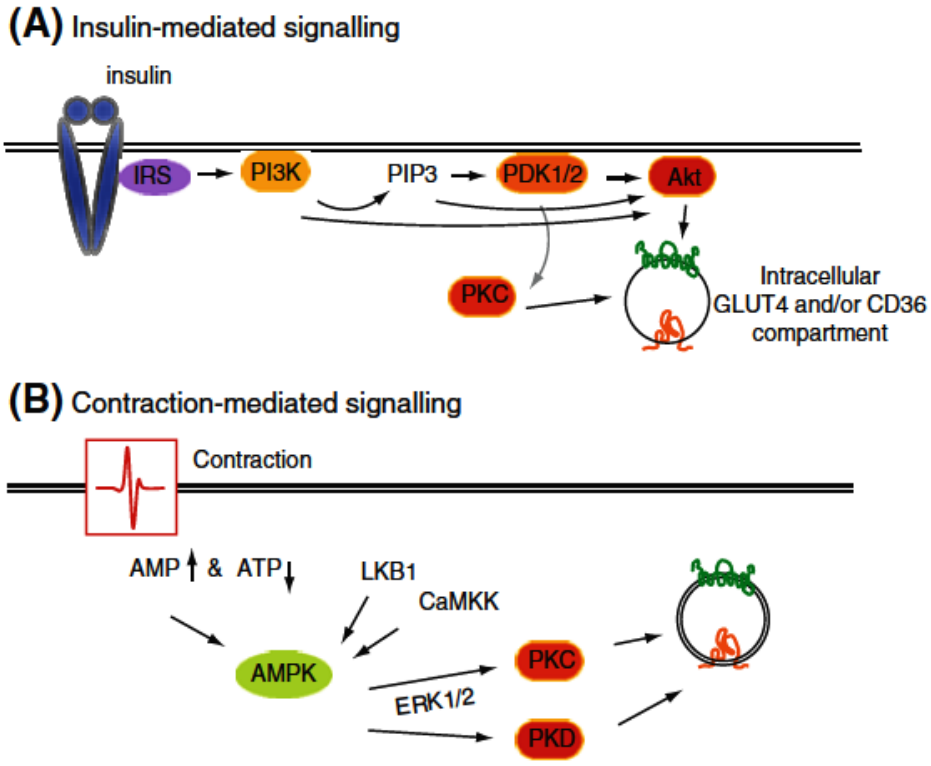

Fig 6: Signaling proteins involved in insulin-stimulated (A) and contraction-stimulated (B) induction of GLUT4 and CD36 trafficking. (A) Upon binding of insulin to its receptor the downstream signaling axis (Akt axis) will be activated which induces the translocation of intracellular GLUT4 and CD36 vesicles. This signaling axis consists of insulin receptor substrate (IRS), phosphatidylinositol-3kinase (PI3K), PI-3 phosphate (PIP3), 3-phosphoinositide-dependent protein kinase (PDK), protein kinase C (PKC) and Akt. (B) Cardiac work increases the AMP/ATP ratio and, subsequently, AMPK activity. For full activation the action of upstream kinases liver kinase B1 (LKB1) or calcium-calmodulin-dependent protein kinase kinase (CaMKK), are wanted. Downstream, and alongside of AMPK, extracellulary regulated protein kinase 1/2 (ERK1/2), PKC and PKD are other players in GLUT and CD36 translocation. Adapted from Steinbusch et al. (2011)

At the present, the knowledge regarding signaling and trafficking processes in CD36 translocation is at its beginning. Although recent finding by Samovski *et al.* (2012) showed that the trafficking of CD36 vesicles to the plasma membrane is regulated by GTP-bound Rab8a protein under the control of the GTPase activation protein AS160, the trafficking of the machinery recruiting CD36 from either insulin or contraction responsive storages is yet to be elucidated.

1.6.4 CD36 in the heart

The heart can use a variety of metabolic substrates for the production of energy, and it has the capacity to rapidly switch among these substrates in response to changes in their supply, hormone stimuli, and the availability of oxygen. During normal conditions, cardiac metabolic energy is provided by FA (60-70%), glucose (20-30%) and lactate (10%). During disease, the balanced utilization of glucose and FA is interrupted and shifts toward either FA utilization (during insulin resistance) or glucose utilization (under hypoxic conditions) (Schwenk *et al.*, 2008).

1.6.4.1 Insulin resistance

Insulin resistance is associated with changes in the lipid metabolism. For example, there is a strong correlation between insulin resistance and plasma FA concentration and intracellular TAG depots (Karpe *et al.*, 2011; Martins *et al.*, 2012). Excessive accumulation of TAG, fatty acyl CoA, DAG and ceramides (referred to as lipotoxicity) causes severe insulin resistance by impairing insulin signaling and multiple steps of glucose metabolism (DeFronzo, 2010). There is growing evidence indicating little or no loss of insulin sensitivity in the heart in type 2 diabetes, while a clear insulin resistance is found in skeletal muscle. However, in diabetes, the heart shifts its substrate utilization almost entirely on FA oxidation to generate ATP and the constant exposure of the heart to high FA levels could exert toxic effects by the generation of noxious derivatives of lipid metabolism. Thus, while the heart may be less susceptible to insulin resistance than skeletal muscle, systemic insulin resistance may have profound negative influence on the myocardium through the toxic effects of substrate overabundance (Chess & Stanley, 2008). It has been shown in the heart of animal models of insulin resistance that the CD36 is permanently translocated to the plasma membrane, which is accompanied with increased

FA uptake (Luiken *et al.*, 2001; Coort *et al.*, 2004; Ouwens *et al.*, 2007). Furthermore, increased FA concentration and accumulation of lipid metabolites is associated with cardiac pathology, which manifests with increased myocyte apoptosis, myocardial fibrosis, left ventricle (LV) hypertrophy, contractile dysfunction and impaired diastolic filling (Wang *et al.*, 2005; Ouwens *et al.*, 2007; Stahrenberg *et al.*, 2010; Falcao-Pires & Leite-Moreira, 2012). The inhibition of CD36 prevents TAG accumulation and thus cardiac dysfunction (Ouwens *et al.*, 2007; Angin *et al.*, 2012). These findings indicate that CD36 is a key factor contributing to the accumulation of intracellular lipids in heart and muscle as well as to the subsequent development of cardiomyopathy in type 2 diabetes. On the other hand, when using CD36 transgenic models, the role of CD36 in insulin resistance is contradictory. Whereas Cd36 knockout mice showed no insulin resistance after intraperitoneal glucose administration (Febbraio *et al.*, 1999), rather increased insulin sensitivity and enhanced glucose uptake and oxidation in muscle were observed (Goudriaan *et al.*, 2003). Others showed insulin resistance induced by high carbohydrate diets in Cd36 knockout mice (Hajri *et al.*, 2002) and SHR with a nonfunctional CD36 (Pravenec *et al.*, 1999). Furthermore, polymorphism in Cd36 has been associated with insulin resistance in humans (Corpeleijn *et al.*, 2006).

1.6.4.2 Cardiac ischemic tolerance

Myocardial I/R injury is a major cause of morbidity and mortality in the western nations (Murphy & Steenbergen, 2008). Myocardial ischemia occurs when coronary blood flow is inadequate, and hence, the oxygen supply to the myocardium is not sufficient to meet the oxygen demand. Under myocardial hypoxic conditions, as caused by an ischemic insult or myocardial infarction (MI), the heart switches to the substrate with the highest oxygen efficiency, i.e. carbohydrates. During reperfusion, when oxygen supply is reinstated, there is a progressive increase in the contribution of FA to ATP production (Stanley *et al.*, 2005). One would expect that the acute substrate utilization switch to carbohydrates during acute ischemia would be accompanied with decreased FA uptake. Instead, the experiments with rat myocytes and perfused hearts showed increased translocation of CD36 to the plasma membrane leading to a concomitant increase in FA uptake (Chabowski *et al.*, 2006). On the other hand, CD36 seems to play an important role

in the recovery of I/R injury, as Irie *et al.*(2003) reported that Cd36 knockout mice showed impaired recovery after MI, which was restored after the rescue of Cd36 gene.

1.6.4.3 Myocardial hypertrophy

The development of cardiac hypertrophy and heart failure is associated with the changes in cardiac substrate preference, namely, glucose utilization is increased at the expense of FA oxidation. This preference have been demostrated in postinfarct heart, where downregulation of genes of lipid metabolic enzymes e.g. acetyl-CoA oxidase, and of FA transporters e.g. Cd36, have been observed (Fliegner *et al.*, 2008). Furthermore, reduced myocardial content of CD36 protein expression with a parallel reduction of palmitate oxidation rate and cardiac ejection fraction has also been observed after MI (Heather *et al.*, 2006). CD36 have been proposed to play an important role in the development of cardiac hypertrophy. Studies on mice given a high-fat diet or aged mice showed that increased CD36 expression contributes to cardiac hypertrophy (Koonen *et al.*, 2007; Sung *et al.*, 2011). However, the studies in genetic animal models showed that a deletion of Cd36 also leads to cardiac hypertrophy (Pravenec *et al.*, 1995; Irie *et al.*, 2003), moreover, genetic variations of the Cd36 have been associated with increased LV mass in humans (Hall *et al.*, 2011). These results suggest that both a shift away from lipid utilization (i.e. hypoxia) and a shift to lipid utilization (i.e. insulin resistance) are associated with the development of cardiac hypertrophy. There is evidence that a change in the balance of myocardial utilization of FA and glucose substrates entail a degree of cellular stress leading to an increased production of ROS, which are known to contribute to cardiac hypertrophy (Mellor *et al.*, 2010).

2 AIMS OF THE THESIS

The fatty acid (FA) transporter CD36 is the main transporter of the heart primary energy source, long-chain fatty acid (LCFA). Disruption of the sensitive balance of long-chain fatty acid and glucose utilization can cause development of heart dysfunction. The SHR possess a mutant Cd36 (Aitman *et al.*, 1999) which causes reduced transport of long-chain fatty acids into the heart and heart hypertrophy (Hajri *et al.*, 2001).

AIM I

This study was based on the knowledge that the SHR is insulin-resistant and that protein kinase C (PKC) isoforms are known to play a role in the insulin signaling cascade as well as in the mechanisms of numerous cardiovascular diseases e.g. heart hypertrophy and heart failure. Thus, the first aim was to study the relationship between CD36-mediated fatty acid transport, activation of two PKC isoforms, δ and ε, and insulin resistance in the heart of the SHR and the congenic strain SHR-4 with an introduced segment of chromosome 4, containing the Cd36 gene, from the insulin sensitive Brown-Norway rats.

Specific aims:

- To compare the concentrations of heart triacylglycerol (TAG) as well as serum glucose, insulin, free FA (FFA), and TAG concentrations in the SHR and SHR-4. Furthermore, to study the effects of a 14-day high-sucrose diet (HSD) feeding on these lipid and glucose metabolites.
- To compare the novel PKC isoforms, δ and ε, expression of the two strains, as well as to analyze the effect of a 14 day HSD feeding on the PKC δ and ε expression and distribution between the cytosol and particulate fraction in the left ventricular of the two strains.

Results published in Klevstig *et al.,* 2011, supplement I

AIM II

A deletion of CD36 has been shown to affect heart I/R injury tolerance (Irie *et al*, 2003). The second aim was therefore to analyze the effect of the nonfunctional CD36 on susceptibility to ischemic ventricular arrhythmias and myocardial infarction in the SHR-4 congenic and SHR-Cd36 transgenic strains and to compare it with the SHR progenitor strain, and to search for potential underlying mechanisms of altered ischemic tolerance.

Specific aims:

- To analyze the incidence and severity of ischemic and reperfusion ventricular arrhythmias and myocardial infarct size (IS) induced by coronary artery occlusion in an open-chest model and in isolated perfused hearts (results published in Neckar *et al.*, 2012, supplement II).
- To study the effect of catecholamine depletion on the incidence and severity of ischemic and reperfusion ventricular arrhythmias and myocardial IS induced by coronary artery occlusion in isolated perfused hearts (results published in Neckar *et al.*, 2012, supplement II).
- To compare the serum free FA and TAG, heart TAG and the FA composition in serum and heart phospholipids of the two strains (results published in Neckar *et al.*, 2012, supplement II).
- To analyze the heart performance after β_1-adrenoceptor stimulation using dobutamine echocardiography stress test in the SHR and SHR-Cd36 (results published in Klevstig *et al*., in press, supplement III).
- To assess the expression and/or functional properties of the major components of the adenylyl cyclase signaling, including β-adrenoceptor, G proteins, adenylyl cyclase and protein kinase A in the left ventricular of both SHR and SHR-Cd36 rats (results published in Klevstig *et al*., in press, supplement III).

3 MATERIAL AND METHODS

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

3.1 ANIMALS

Adult SHR, SHR-4 and SHR-Cd36 (from the Institute of Physiology, Czech Academy of Science breeding station) were kept in at a 12 h: 12 h light/dark period with free access to standard laboratory chow and water, unless otherwise indicated. The experiments were performed in agreement with the Animal Protection Law of the Czech Republic (311/1997), and were approved by the Ethics Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic.

3.2 AIM I

3.2.1 Diet

The 5 months old SHR and SHR-4 rats were divided into two groups 14 days before sacrifice, control and experimental. The control groups were fed a standard laboratory chow (SEMED, Prague, Czech Republic) and the experimental groups were fed a high-sucrose diet containing, in caloric %: 60 % sucrose, 20 % proteins and 20 % fats.

3.2.2 Tissue preparation

The animals designed for PKC determination were killed by decapitation, and their hearts were rapidly excised, washed in cold (0 °C) saline, and dissected into the left and right free ventricular walls and the septum on ice dish. All parts were frozen in liquid nitrogen, weighed separately, and stored in liquid nitrogen until use.

3.2.3 Tissue fractionation for analysis of PKC isoforms expression

Frozen LV myocardium was pulverized to a fine powder at the temperature of liquid nitrogen, followed by Potter-Elvehjem homogenization in eight volumes of ice-cold buffer

composed of (in mmol/l) 12.5 Tris (pH 7.4), 250 sucrose, 2.5 EGTA, 1 EDTA, 100 NaF, 5 DTT, 0.3 phenylmethylsulfonyl fluoride, 0.2 leupeptin, and 0.02 aprotinin. The homogenate was centrifuged at 100,000 x *g* for 90 min. The resulting pellet represented the particulate fraction; the supernatant was the cytosolic fraction. The pellet of the particulate fraction were resuspended in homogenization buffer containing 1 % Triton X-100, held on ice for 90 min, and then centrifuged at 100,000 x *g* for further 90 min. The resulting detergent-treated supernatants were used for immunoblot analyses. Triton X-100 was added to the cytosolic fraction to reach the final concentration of 1 %. The samples were stored at −80 °C until analysis. Protein content was determined according to Lowry modified by Peterson (1977).

3.2.4 Denaturing (SDS) discontinuous gel electrophoresis

Electrophoretic protein separations were performed in vertical slab (Mini–PROTEAN 3 Cell system, Bio-rad®) polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) according to Ogita & Market (1979), using a 4 % stocking gel and a 10 % separating gel. Samples were diluted in sample buffer, boiled for 3 min and loaded in to the cells. 10 mA/gel was used during the migration through the stocking gel and then increased to 25 mA/gel throughout the separating gel.

3.2.5 Western Blot analysis of PKC isoforms

After electrophoresis, the gels were incubated in transfer buffer for 5 min and then layered between gel blot papers together with nitrocellulose membrane (Amersham Biotechnology). The electroblot was performed at 350 mA for 60 min.

Membranes were incubated in 5 % dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 60 min at room temperature to block nonspecific protein binding. After being washed in TTBS buffer (3 times quickly, 3 x 5 min each), the membranes were probed with specific primary rabbit antisera (Sigma; PKC δ (1:8000), PKC ε (1:8000), β-actin (1:5000)) for 90 min at room temperature. The membranes were washed again and incubated with the secondary swine anti-rabbit antibody labeled with horseradish peroxidase (Sigma; 1:6000) for 60 min at room temperature. Before enhanced chemiluminescence (ECL), the membranes were washed as described above and stored in

TTBS for at least 2 h. For ECL, luminol and H_2O_2 solutions were prepared, mixed 1:1 just before use, and poured on membranes. The specific signal was detected on an autoradiographic film (Amersham Biotechnology). Scanning (Epson Perfection 1240U Scanner) and ImageQuant software were used for quantification of the relative abundance of individual PKC isoforms. The amount of protein applied to the gel varied for each isoform and fraction to achieve linearity with the intensity x area (volume) of the band on the Western blot. To ensure the specificity of the immunoreactive proteins, prestained molecular weight protein standards (Sigma, Bio Rad), rat brain extract, recombinant and human PKC δ and PKC ε standards (Sigma) as well as the respective PKC blocking immunizing peptide (Sigma) were used. β-Actin was used for comparative quantification of the monitored protein amount in Western blot analysis.

3.2.6 Heart TAG

For determining heart TAG concentration, heart tissue was powdered under liquid nitrogen and extracted overnight in chloroform-methanol mixture (2:1 v/v), after which 20 % volumes of 2 % KH_2PO_4 were added and the solution was centrifuged. The organic phase was removed and evaporated under N_2. The resulting pellet was dissolved in isopropyl alcohol, and the TAG content was determined using a standard enzymatic technique (Pliva-Lachema, Brno, Czech Republic).

3.2.7 Serum glucose, insulin, free FA and TAG

Blood was taken from the tail into 5 % trichloroacetic acid and promptly centrifuged (3000 x *g*, 10 min) at 4 °C and the serum was stored frozen at −70 °C until analysis.

The blood glucose concentration was measured by enzymatic method after trichloroacetic acid protein precipitation followed by oxidation by glucose oxidase assay kit (Pliva-Lachema, Brno, Czech Republic).

Serum insulin concentration was determined using Mercodia Rat Insulin ELISA kit (Mercodia AB, Uppsala, Sweden), the serum free FFA were measured enzymatically after the conversion of FFA to acyl-CoA catalyzed by acyl-CoA synthase followed by acyl-CoA oxidase oxidation by an analytic kit (Roche Diagnostics, Basel, Switzerland) and TAG

were measured enzymaticlly after lipoprotein lipase hydration using a colorimetric kit (Pliva-Lachema, Brno, Czech Republic).

3.2.8 Statistical analysis

All data are expressed as mean ± SEM. Two-way analysis of variance and subsequent Student-Newman-Keul's test was used for comparing the differences in normally distributed variables among the group. Differences were taken to be statistically significant when $P<0.05$.

3.3 AIM II

3.3.1 Infarct size and arrhythmias in open chest rats

The infarcts and arrhythmias were performed under the supervision of Jan Neckář, PhD., Institute of Physiology, AS CR.

Anesthetized 4 month old SHR, SHR-4 and SHR-Cd36 rats (pentobarbital sodium, 60 mg/kg i.p.; Sigma-Aldrich, USA) were ventilated (rodent ventilator 7026, UgoBasile, Italy) *via* tracheal cannula with room air at 68 strokes/min (total volume of 1.2 ml/100 g body weight). Both blood pressure in the left carotid artery and a single-lead electrocardiogram (ECG) were continually recorded. The rectal temperature was maintained between 36.5 and 37.5 °C by a heated table throughout the experiment. Left thoracotomy was performed and after 10 min stabilization, regional myocardial ischemia was induced by tightening a polyester ligature 6/0 (Ethicon, Edinburgh, UK) placed around the left anterior descending coronary (LAD) artery about 1-2 mm distal to its origin. Characteristic changes in the configuration of the ECG and a transient decrease in blood pressure verified the coronary artery occlusion. After a 20 min occlusion period, the ligature was released and reperfusion of previously ischemic tissue continued.

At the end of 3 h reperfusion, the hearts were excised and washed with 20 ml saline through the cannulated aorta. The LAD coronary artery was then reoccluded and the ischemic zone was delineated by staining the non-ischemic tissue with 5 % potassium

permanganate (Lachema, Czech Republic) dissolved in water (2 ml through the aorta). After washing out the solution thoroughly with saline and releasing the occlusion, the hearts were perfused with 1 % 2,3,5-triphenyltetrazolium chloride (TTC) dissolved in 0.1 mol/l phosphate buffer (pH 7.4) to stain surviving myocardium of the area at risk (AR). The hearts were then incubated in the effluent of TTC for 20 min. The hearts were cut perpendicularly to the long axis of the ventricle into slices 1 mm thick and stored overnight in 10 % neutral formaldehyde solution. The day after the IS staining, the right ventricular free wall was separated and both sides of the slices were photographed. The IS, the size of the AR and the size of the LV were determined by a computerized planimetric method using the software Ellipse (ViDiTo, Slovakia) with a grid of about 400 points per slice. The IS was normalized to the AR (IS/AR) and the AR was normalized to the LV (AR/LV).

Incidence and severity of ventricular arrhythmias during ischemic insult and the first 5 min of reperfusion were assessed. Premature ventricular complexes (PVCs) occurring as singles, salvos or tachycardia (a run of 4 or more consecutive PVCs) were counted separately. The incidence of ventricular tachycardia (VT) and fibrillation (VF) was also evaluated. VF lasting more than two minutes was considered as sustained (VFs).

3.3.2 Infarct size and arrhythmias in isolated perfused hearts

SHR and SHR-Cd36 were anesthetized as described above (chapter 3.3.1). Hearts were rapidly excised and perfused at constant flow (~10 ml/min/g) and temperature (37 °C) according to Langendorff under non-recirculating conditions by a modified Krebs-Henseleit solution (mmol/l: NaCl 118.0, KCl 3.2, $CaCl_2$ 1.25, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2, glucose 7.0). The medium was gassed with 95 % O_2 and 5 % CO_2 (pH 7.4). The expected heart weights were calculated from regression equations established on the basis of our previous data for heart weight to body weight ratio of SHR and SHR-Cd36.

Epicardial electrograms were continuously recorded and subsequently analyzed by custom designed software. After 20 min stabilization, regional no-flow ischemia was induced by the LAD occlusion for 40 min followed by 1 h reperfusion. Ventricular arrhythmias during ischemia and the first 10 min of reperfusion were counted and the IS determined similarly as in open-chest experiments.

The involvement of catecholamines in cardiac ischemic tolerance of SHR and transgenic SHR-Cd36 hearts was examined in rats pretreated with reserpine (alkaloid that

depletes catecholamines; 0.15 mg/kg dissolved in a mixture of glacial acetic acid and saline 1:50) administered i.p. 24 h before ischemia according to Oxman *et al.* (1997). Control rats received the same volume of the vehicle.

3.3.3 FA composition in serum and membrane phospholipids

The HPLC analysis of the FA was performed by Marek Vecka PhD., First Faculty of Medicine, Charles University in Prague, CR.

Serum (1 ml) was dissolved in 21 ml of a chloroform-methanol mixture (2:1, v/v) and shaken in a pear-shaped flask. The serum protein precipitate was removed by filtration. The extract was centrifuged (5 min, 2000 rpm, 4 °C) after the addition of saline (20 % of the extract volume). Then 12 ml of a chloroform-methanol-water mixture (3:48:47, v/v) was added to the lower phase and after a vigorous shaking and centrifugation (5 min, 2000 rpm, 4 °C), the lower lipid layer was carefully separated and evaporated at 40 °C under a stream of nitrogen.

200 mg of frozen LV myocardium was pulverized to a fine powder at the temperature of liquid nitrogen. Phospholipids were extracted from tissue samples according to the modified method of Folch *et al.* (1957) in three consecutive steps. The first extraction was performed in three portions (0.5 ml each) of chloroform-methanol mixture (1:3, 2:1 and 2:1, v/v) in a chilled mortar. The mixture was brought to test tubes and centrifuged (10 min, 3000 rpm, 4 °C), the supernatant (extract) was saved while the pellet was resuspended in chloroform-methanol mixture (2:1, v/v, 1.25 ml) and centrifuged (10 min, 3000 rpm, 4°C). Resulting supernatant was added to the extract and the pellet was processed as above. Saline (20 % of the extract volume) was added to the extract, vigorously shaken and centrifuged (10 min, 3000 rpm, 4 °C). The lower lipid layer was carefully separated and evaporated at 40 °C under a stream of nitrogen.

Serum and membrane lipid classes were separated by thin layer chromatography using hexane-diethylether-acetic acid (85:15:1, v/v) as a solvent system. Silica Gel H (Merck, Darmstadt, Germany) as a slurry of 22.5 g in 62 ml water containing 2.5 g of magnon (Merck) was spread in a 0.25 mm layer with a spreader (Desaga, Heidelberg, Germany) on glass plates (20 × 20 cm). For FA analyses, phospholipid spots were visualized under UV light, scraped out and stored under nitrogen atmosphere at –20 °C until the next day when

methyl esters were prepared. For FA methyl ester preparation, sodium methanolate was added to tubes with silica gel; tubes were then incubated for 60 min at room temperature in dark. Methyl esters were extracted with hexane, the extract was evaporated under a stream of nitrogen and stored at –20 °C. Gas chromatography analysis of the FA methyl esters was performed as previously described (Tvrzicka *et al.*, 2002) on a gas chromatograph GC-17A (Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and automatic sampler AOC-20i (Shimadzu, Kyoto, Japan). A fused silica capillary column (30 m x 0.32 mm ID) with chemically bonded stationary phase DB-WAXETR from J&W Scientific (Folsom, CA, USA) was used. The oven temperature was maintained for 1 min at 80 °C, then raised to 150 °C by 20 K/min, then by 3 K/min to 250 °C and kept for 5 min; hydrogen was used as the carrier gas. Injector and detector temperatures were set at 250 °C. Individual peaks of FA methyl esters were identified by comparing the retention times with those of authentic standards (Nu-Chek Prep Inc., Elysian, MN, U.S.A.). The composition of serum and LV FA (spectrum of 27 main FA) was analyzed. The product/precursor ratios of the FA were used to calculate indices reflecting the activities of enzymes involved in FA metabolism: elongase (18:0/16:0), Δ6 desaturase (18:3n8/18:2n6), Δ5 desaturase (20:4n6/20:3n6), and Δ9 desaturase (16:1n7/16:0).

3.3.4 Serum FFA and TAG and heart TAG

Serum FFA and TAG and the heart TAG in the SHR and SHR-Cd36 rats were analyzed as described above (chapter 3.2.7 and 3.2.8).

3.3.5 Dobutamine stress echocardiography

The dobutamine test was performed by František Papoušek, Institute of Physiology, AS CR.

Echocardiographic measurements were performed using GE Vivid 7 Dimension (GE Vingmed Ultrasound, Horten, Norway) with 12 MHz linear matrix probe M12L. Animals were anesthetized by inhalation of 2 % isoflurane (Aerrane, Baxter SA) and their rectal temperature was maintained within 36.5 and 37.5 °C by a heated table throughout measurements. After the acquisition of baseline data, dobutamine was serially infused (i.v.) at rates of 1, 5 and 25 ng/g/min for 4 min each. The following diastolic and systolic

dimensions of the LV were measured using M-mode in long-axis view: posterior wall thickness (PWT_D and PWT_S), anterior wall thickness (AWT_D and AWT_S), and cavity diameter (LVD_D and LVD_S). The main functional parameter, fractional shortening (FS%) was derived by the following formula:

$$FS\% = \frac{LVD_D\text{-}LVD_S}{LVD_D} \times 100$$

3.3.6 Crude myocardial membrane preparation

The characterization of the β-ARs, G proteins and AC was performed in cooperation with Dmitry Manakov, Faculty of Science, Charles University in Prague, CR.

Frozen samples of the LV were placed into 10 volumes ice-cold TMES buffer (mmol/l: Tris 20, $MgCl_2$ 3, EDTA 1, sucrose 250; pH 7.4) containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), cut into small pieces and homogenized in an Ultra-Turrax blender (15 s). The resulting suspension was further homogenized for 1 min using a motor-driven teflon pestle in a glass tube homogenizer, and then centrifuged (600 x *g*, 10 min, 4 °C). The resulting supernatant was collected and the pellet resuspended in TMES buffer and centrifuged (600 x *g*, 10 min, 4 °C). The two supernatants obtained in the previous centrifugation steps were mixed and centrifuged (50 000 x *g*, 30 min, 4 °C) and the resulting pellet (crude membrane fraction) was resuspended in TME buffer (mmol/l: Tris 20, $MgCl_2$ 3, EDTA 1; pH 7.4). Aliquots were snap-frozen and stored at −80 °C until analysis. Protein concentration was determined according to the Bradford colorimetric method (Bradford, 1976).

3.3.7 β-Adrenoceptor binding

Myocardial β-AR density and affinity were determined by specific binding of the β-AR antagonist [^{3}H]CGP-12177 (Amersham Biosciences, Buckinghamshire, UK). Samples of myocardial crude membranes (100 µg protein) were incubated in buffer A (mmol/l: Tris-HCl 50, $MgCl_2$ 10, ascorbic acid 1; pH 7.4) containing increasing concentrations of [^{3}H]CGP-12177 (0.09375-3 nmol) for 1 h at 37°C in a total volume of 0.5 ml. The binding reaction was terminated by addition of 3 ml ice-cold buffer B (mmol/l:

Tris-HCl 50, $MgCl_2$ 10; pH 7.4) and subsequent filtration through GF/C filters (Whatman Ltd., Oxford, UK) presoaked for 1 h with polyethylenimine. The filters were washed twice with 3 ml of ice-cold buffer B. Radioactivity retained on the filters were determined by liquid scintillation counting using CytoScint cocktail (ICN Biomedicals Irvine, CA, USA). Nonspecific binding was defined as that not displaceable by 10 µmol/l L-propranolol, and it represented < 25 % of total binding. For competition isotherms, samples of crude myocardial membranes were incubated with 1.5 nmol/l [^{3}H]CGP-12177 and increasing concentrations of ICI118.551 (4-10 nmol/l), a selective β_2-AR antagonist. The characteristics of β-adrenergic binding sites and the β_1- and β_2-AR proportions in myocardial membranes were calculated using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

3.3.8 Determination of adenylyl cyclase activity

AC activity was determined in crude membranes prepared from the LV of SHR-Cd36 and SHR rats. The reaction mixture (in a total volume of 100 µl) containing (in mmol/l) 50 Tris-HCl (pH 7.4), 2 $MgCl_2$, 20 GTP, 0.040 ATP, 100 NaCl, 0.8 mg/ml BSA, 0.040 3-isobutyl-1-methylxanthine, 5 potassium phophoenolpyruvate and 32 units of pyruvate kinase. The reaction was initiated by the addition of 10 µl of ATP and the assay was run for 20 min at 30 °C. The following different stimulators were used in separate experiments: forskolin (10 µmol/l), GTPγS (10 µmol/l), isoproterenol (10 µmol/l), or $MnCl_2$ (10 µmol/l). The reaction was terminated by adding 200 µl 0.15 mol/l HCl and the levels of cAMP were determined using a cAMP EIA kit (NewEastBioscienses, Inc., Malvern, PA, USA).

3.3.9 Cardiac tissue preparation for PKA analysis

Frozen LV myocardium was pulverized to a fine powder under a stream of liquid nitrogen, followed by Potter-Elvehjem homogenization in eight volumes of ice-cold buffer (mmol/l: Tris 12.5, sucrose 250, EGTA 2.5, EDTA 1, NaF 100, 2-mercaptoethanol 6, phenylmethylsulfonyl fluoride 0.3, leupeptin 0.2, aprotinin 0.02; pH 7.4). Aliquots of the homogenate were stored at −80 °C until analysis. Protein concentration was determined according to the Bradford colorimetric method (Bradford, 1976).

3.3.10 Electrophoresis and immunoblotting of G protein, adenylyl cyclase and PKA

Electrophoresis and immunoblotting were performed as described above (chapter 3.2.5 and 3.2.6). Briefly, samples of myocardial preparations resolved by SDS-polyacrylamide gel electrophoresis using 10 % gels were transferred onto the nitrocellulose membrane (Schleicher-Schuell, Erdmannhause, Germany) and then incubated in 5 % non-fat dry milk in TTBS buffer for 1 h at room temperature. After washing with TTBS buffer (3 x 5 min), the membranes were incubated with specific polyclonal antiserum (all antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 90 min at room temperature. The membranes were washed again (3 x 5 min) and then incubated with horseradish peroxidase conjugated secondary antibodies (Sigma, St. Louis, MO, USA) for 1 h. Immunoreactive proteins were made visible using enhanced chemiluminescence and quantitatively analyzed by the ImageQuant program (Molecular Dynamics, Sunnyvale, CA, USA). The distribution of β-actin (as an internal loading standard) was monitored for reliable determination quantitative changes in proteins detected by Western blot.

3.3.11 Statistical analysis

Data are expressed as means ± SEM. Differences in parametric variables between the groups were compared by unpaired Student t-test. Differences in the number of PVCs were compared by the Mann-Whitney U test. The incidence of tachyarrhytmias was examined by Fisher's exact test. Statistical significance was defined as $P<0.05$.

4 Results

4.1 AIM I

SHR is insulin-resistant strain and PKC isoforms are known to play a role in the insulin signaling cascade as well as in the mechanisms of numerous cardiovascular diseases e.g. cardiac hypertrophy and heart failure. The first aim was therefore to study the relationship between CD36 mediated FA transport, activation of two PKC isoforms, δ and ε, and insulin resistance in the heart of the SHR and the congenic strain SHR-4 that have a segment of chromosome 4, containing the Cd36 gene, from the insulin sensitive BN.

4.1.1 Effect of HSD on heart weight and lipid and glucose metabolites

Strain differences

The congenic SHR-4 strain had lower systolic blood pressure when compared to the SHR. Both the SHR and SHR-4 strains had similar body weights (BW) (Table 1).

Table 1: Blood pressure, body and heart weight, serum lipids and insulinemia of SHR and SHR-4 rats fed control or HSD diets

	Control		HSD	
	SHR	SHR-4	SHR	SHR-4
n	10	9	10	9
SBP, mmHg	173 ± 3	159 ± 3*	NA	NA
BW, g	418 ± 4	427 ± 9	435 ± 7	442 ± 6
HW/BW, mg/g	3.64 ± 0.17	2.89 ± 0.04*	3.38 ± 0.06	3.02 ± 0.07*
LV/BW, mg/g	2.28 ± 0.09	1.89 ± 0.04*	1.98 ± 0.10$^{\#}$	1.89 ± 0.07
Glucose, mmol/l	5.82 ± 0.12	5.78 ± 0.14	7.35 ± 0.33$^{\#}$	5.71 ± 0.15
Insulin, pmol/l	224 ± 44	119 ± 24*	305 ± 43	209 ± 44$^{* \#}$
Serum FFA, mmol/l	0.51 ± 0.06	0.18 ± 0.02*	0.87 ± 0.11$^{\#}$	0.31 ± 0.03*
Serum TAG, mmol/l	0.80 ± 0.01	0.68 ± 0.03	1.43 ± 0.04$^{\#}$	0.96 ± 0.06$^{* \#}$

*Values are means ± SEM; spontaneously hypertensive rat (SHR), SHR congenic strain (SHR-4) number of animals used (n), systolic blood pressure (SBP), not analyzed (NA), body weight (BW), high sucrose diet (HSD), heart weight (HW), left ventricle (LV), free fatty acid (FFA), triacylglycerols (TAG), *P<0.05 SHR-4 vs. corresponding SHR; $^{\#}$P<0.05 HSD vs. corresponding Control.*

While the relative weights of the heart (HW/BW) and of the LV (LV/BW) were significantly lower in the SHR-4 compared to the SHR. Circulating levels of insulin and FFA in the SHR-4 were 53 % and 35 % of those in SHR. Meanwhile, no differences in serum glucose and TAG concentrations were observed between the two strains (Table 1).

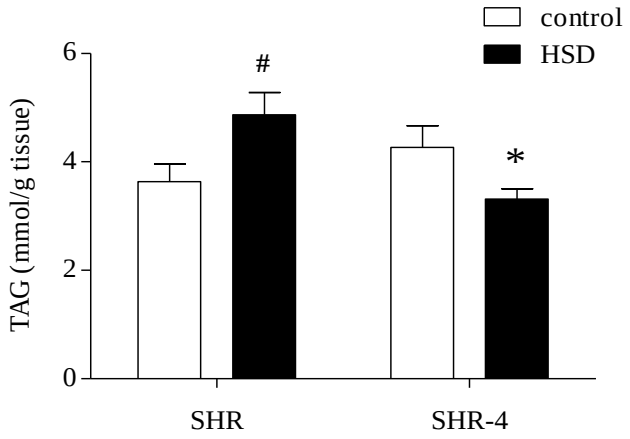

Fig 7: Triacylglycerol (TAG) concentration in hearts from SHR and SHR-4 rats fed either a control or high sucrose diet (HSD). Values are means ± SEM; $^{*}P<0.05$ SHR-4 vs. corresponding SHR; $^{\#}P<0.05$ HSD vs. corresponding Control.

Effect of high-sucrose diet

The HSD suppressed the relative LV weight in SHR to 86 % of its control. Moreover, HSD increased the serum glucose, FFA and TAG by 26 %, 71 % and 79 % (Table 1), respectively, and the heart TAG (Fig 7) by 36 % of the control values. In SHR-4, the HSD increased the serum concentrations of insulin and TAG by 76 % and 41 %, respectively, whereas the relative LV weight, serum glucose level (Table 1) and heart TAG (Fig 7) were not significantly influenced in comparison with control SHR.

4.1.2 Effect of HSD on novel PKC isoform expression

Immunoreactivities of PKC δ and PKC ε were detected on Western blots as single and double bands, respectively. These were confirmed by the respective blocking peptide and a positive control from rat brain homogenate extract (Fig 8A and 8B).

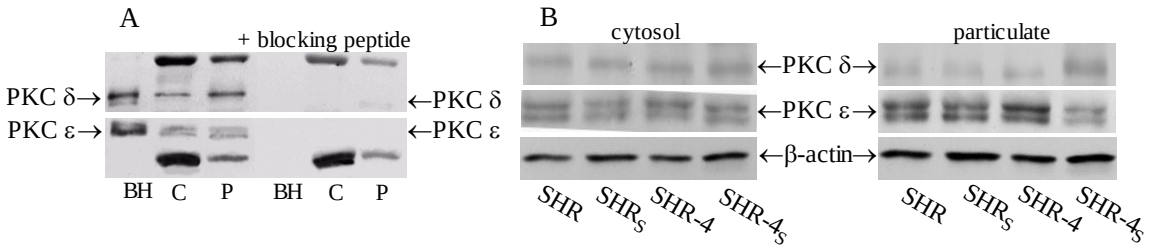

Fig 8: Representative Western blots of PKC δ and PKC ε in cytosolic (C) and particulate (P) fractions from LV of Wistar rats and rat brain homogenate (BH) in the absence (left) and presence (right) of the respective blocking peptide (A). Representative Western blots of PKC δ, PKC ε and the loading control, β-actin, in cytosolic and particulate fractions from SHR and SHR-4 control or after high sucrose diet (marked by subscript S)(B).

Strain difference

The comparison of both PKC isoforms between the strains demonstrated that there was no difference in the PKC δ expression neither in the cytosolic nor particulate fractions (Fig 9A). However, the PKC ε expression was higher in the particulate fraction of the SHR-4 compared with the SHR (Fig 9C). Furthermore, the particulate/total ratios (activated forms proportion) PKC ε were higher in the SHR-4 (Fig 9D).

Effect of high sucrose diet

The HSD did not influence the expression of either PKC ε or PKC δ in SHR. In the SHR-4, however, the HSD increased the expression of PKC δ and decreased the expression of PKC ε in the particulate fraction (Fig 9A and 9C). Despite the HSD-induced changes in the PKC expression in the particulate fraction, neither the PKC δ nor PKC ε particulate/total ratios were affected (Fig 9B and 9D).

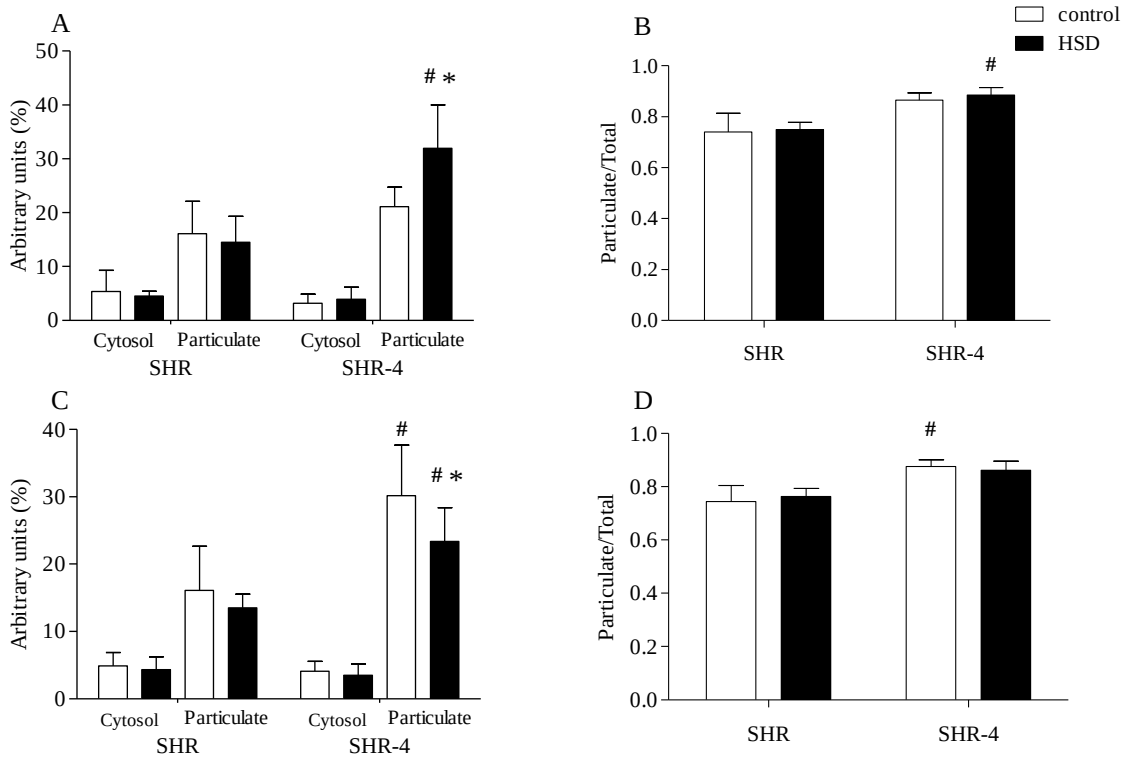

*Fig 9: PKC δ and ε expression in left ventricle of SHR and SHR-4 rats fed either a control or high sucrose diet (HSD) (A, C). PKC δ and ε particulate/total (cytosol+particulate) ratio in SHR and SHR-4 rats fed either a control or HSD (B, D). Values are means ± SEM; *P<0.05 SHR-4 vs. corresponding SHR; #P<0.05 HSD vs. corresponding Control.*

4.2 AIM II

After studying PKC and insulin resistance in the SHR-4 congenic strain and the SHR we decided to examine the tolerance to I/R injury in these strains. We found a tendency to a smaller IS as well as higher amount of PVCs in the SHR-4 when compared to the SHR (Fig 10A and 10B). Since the differences between the strains were not significant, we choose to compare another CD36 genetically modified model, SHR-Cd36, which is 100 % genetically identical to the SHR progenitor strain, except the wild-type Cd36.

The aim of the study was to analyze the effects of mutant Cd36 on susceptibility to ischemic ventricular arrhythmias and MI in SHR-Cd36 transgenic rats with wild type Cd36 compared to age-matched SHR controls and to search for possible molecular mechanisms of altered ischemic tolerance.

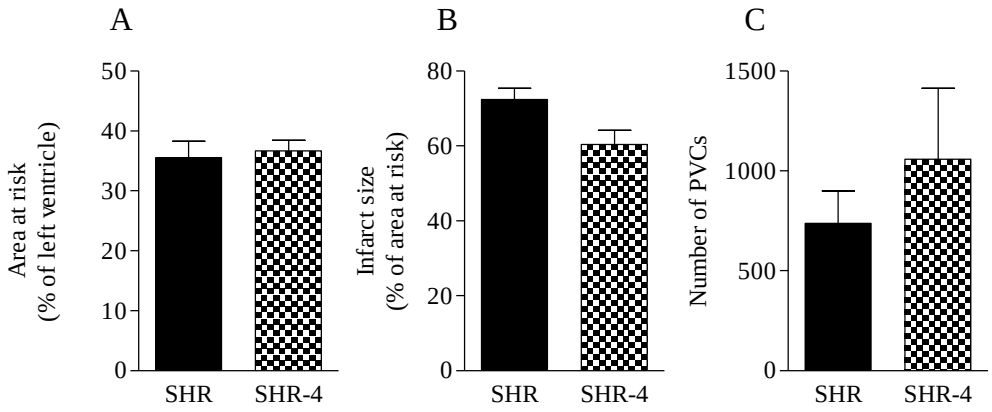

*Fig 10: Size of area at risk expressed as percent of the left ventricle (A), myocardial infarct size expressed as percent of the area at risk (B) and the total number of premature ventricular complexes (PVCs) (C) in control SHR and congenic SHR-4 rats. Values are means ± SEM; *P<0.05 vs. SHR, ǂP<0.05 vs. SHR-4.*

4.2.1 Weight parameters of SHR and SHR-Cd36

Blood pressure, body and heart weight parameters of SHR and SHR-Cd36 rats are summarized in Table 2. We confirmed that the transgenic SHR-Cd36 strain had lower systolic blood pressure when compared to the SHR. There was no difference in body weight, whereas SHR-Cd36 exhibited higher total heart and LV weights. Likewise, the relative heart and LV weight was higher in SHR-Cd36 than SHR.

Table 2: Blood pressure, body and heart parameters of the SHR and SHR-Cd36

Strain	n	SBP, mmHg	BW, g	HW, mg	LV, mg	HW/BW, mg/g	LV/BW, mg/g
SHR	16	198 ± 2	307 ± 4	0.99 ± 0.01	0.574 ± 0.009	3.21 ± 0.04	1.87 ± 0.03
SHR-Cd36	16	186 ± 3*	308 ± 4	1.04 ± 0.01*	0.611 ± 0.008*	3.38 ± 0.02*	1.98 ± 0.01*

*Values are means ± SEM; spontaneously hypertensive rat (SHR), CD36 transgenic SHR (SHR-Cd36), number of animals (n), systolic blood pressure (SBP),body weight (BW), heart weight (HW), left ventricle (LV); *P<0.05 vs. SHR.*

4.2.2 Infarct size and ventricular arrhythmias in open-chest rats.

Fig 11A shows the representative examples of myocardial infarction in SHR and SHR-Cd36 rats. The normalized area at risk did not differ between the strains (Fig 11B). The expression of Cd36 transgene was associated with a significant reduction of

myocardial IS to 52.6 ± 4.3 % of the area at risk compared to 72.4 ± 2.9 % in SHR controls (Fig 11C).

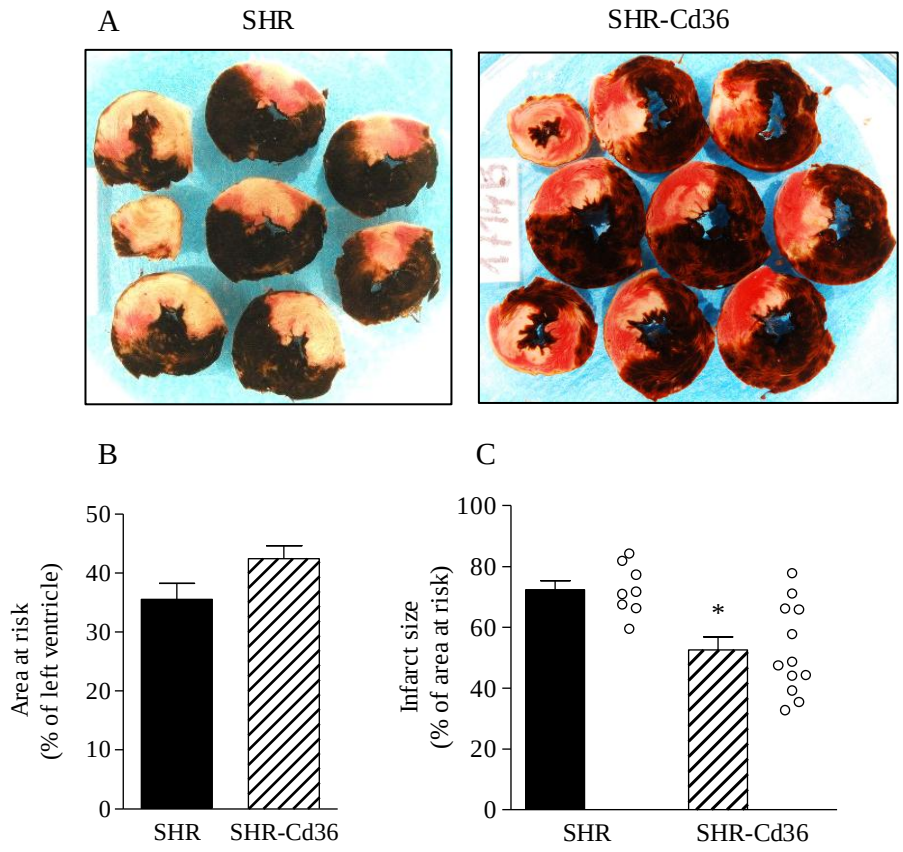

Fig 11: Typical examples of myocardial infarction (infarct area – white; surviving tissue – red; perfused tissue – brown) (A), size of area at risk expressed as percent of the left ventricle (B), and myocardial infarct size expressed as percent of the area at risk (C) in control SHR and transgenic SHR-CD36 rats. Open circles show individual measurements. Values are means ± SEM; $^{*}P<0.05$ vs. SHR.

In contrast to MI, transgenic expression of Cd36 was associated with a marked increase in the incidence and severity of ischemic ventricular arrhythmias. In both groups, VT was the predominant form of ischemic arrhythmias. Expression of Cd36 transgene dramatically increased the total duration of VT episodes (196 ± 47 s vs. 55 ± 21 s) and the number of PVCs occurring as VT (2136 ± 508 vs. 527 ± 191) when compared to SHR controls. This greater susceptibility to VT substantially contributed to the increase in total number of PVCs and duration of tachyarrhythmias (VT + VF) during the ischemic insult (Fig 12A and 12B). Ventricular arrhythmias occurring in the early phase (first 5 min) of

reperfusion were also significantly promoted in the SHR-Cd36 group as indicated by the increased number of total PVCs (215 ± 76 s vs. 56 ± 22) (Fig 12C).

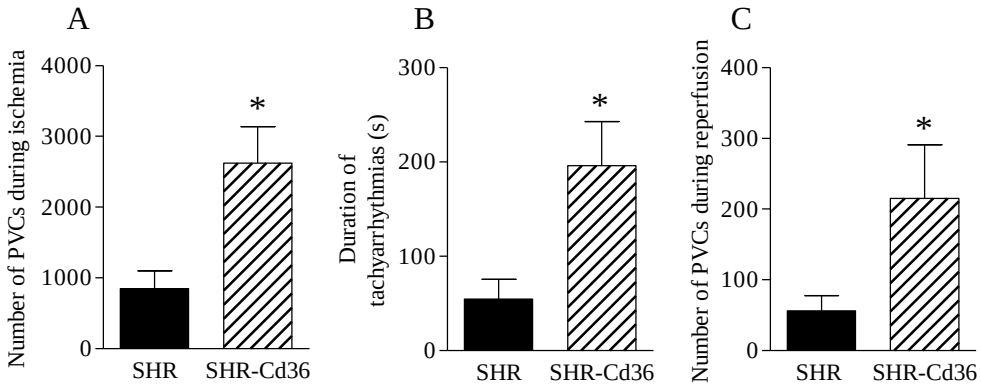

Fig 12: The total number of ischemic premature ventricular complexes (PVCs) (A), duration of ischemic tachyarrhythmias (B) and PVCs at the beginning of reperfusion (C) in control SHR and transgenic SHR-Cd36 rats. Values are means ± SEM; $^{*}P<0.05$ vs. SHR.

4.2.3 Infarct size and arrhythmias in isolated perfused hearts and the effect of reserpine

Proarrythmic and IS limiting phenotype of transgenic rats was also confirmed in perfused hearts. Similarly to the myocardial IS *in situ*, the expression of Cd36 transgene was associated with a significant reduction of IS to 6.76 ± 1.4 % of the area at risk in SHR-Cd36 compared to 19.0 ± 3.2 % in SHR controls. Reserpine did not affect the IS in either group (Fig 13A).

The total number PVCs were markedly increased in transgenic rats (1057 ± 360) as compared to SHR (271 ± 87), although this difference did not reach significance due to a high individual variability. Depletion of catecholeamines by reserpine completely abolished the increased susceptibility to ischemic arrhythmias in SHR-Cd36 as documented by a reduced total number of PVCs (Fig 13B).

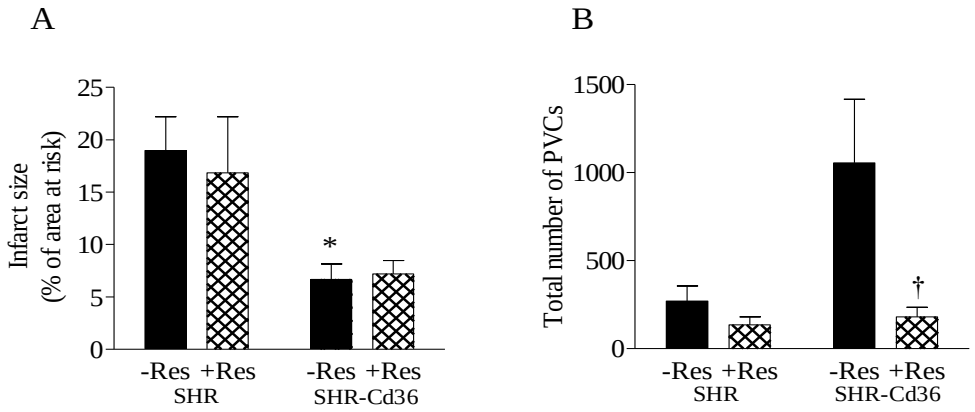

*Fig 13: Myocardial infarct size expressed as percent of the area at risk (A) and the total number of premature ventricular complexes (PVCs) (B) in isolated perfused hearts of untreated (-Res) and reserpine-pretreated (+Res) control SHR and transgenic SHR-Cd36 rats. Values are means ± SEM; *P<0.05 vs. SHR, †P<0.05 vs. corresponding untreated control.*

4.2.4 Metabolic traits

As can be seen in Table 3, the expression of the Cd36 transgene was associated with significantly lower levels of serum FFA and TAG. In addition, heart TAG concentration was also significantly lower in transgenic rats when compared to SHR controls.

Table 3: The levels of serum FFA and TAG and cardiac TAG in SHR and SHR-Cd36

	Serum		Cardiac
Strain	FFA, mmol/l	TAG, mmol/l	TAG, µmol/g
SHR	0.540 ± 0.047	0.776 ± 0.058	2.63 ± 0.26
SHR-Cd36	0.419 ± 0.028*	0.601 ± 0.047*	1.88 ± 0.24*

*Values are means ± SEM; spontaneously hypertensive rat (SHR), CD36 transgenic SHR (SHR-Cd36), free fatty acids (FFA), triacylglycerol (TAG); *P<0.05 vs. SHR.*

The two strains did not differ in the proportion of major FA in heart phospholipids (Table 4). On the contrary, in serum phospholipids, a lower level of vaccenic acid (18:1n-7) and arachidonic acid (20:4n-6) was observed in the SHR-Cd36 rats as compared with SHR controls. The indices of FA enzyme (elongase and desaturase) activities in serum and heart did not differ between groups (Table 4).

Table 4: Fatty acid composition and indices of fatty acid enzyme activities estimated from serum and heart phospholipids measurements in SHR and SHR-Cd36

	Serum		Heart	
Fatty acid	SHR	SHR-Cd36	SHR	SHR-Cd36
16:00	26.64 ± 0.56	27.84 ± 0.18	11.52 ± 0.19	12.02 ± 0.22
18:00	17.50 ± 0.43	18.04 ± 0.38	21.71 ± 0.12	21.74 ± 0.13
18:1n-9	3.65 ± 0.13	3.58 ± 0.03	2.25 ± 0.04	2.23 ± 0.07
18:1n-7	2.16 ± 0.05	1.98 ± 0.03*	3.56 ± 0.06	3.53 ± 0.07
18:2n-6	21.23 ± 0.30	22.58 ± 0.57	28.99 ± 0.47	29.35 ± 0.48
20:4n-6	17.14 ± 0.57	15.44 ± 0.34*	15.14 ± 0.23	14.43 ± 0.31
22:5n-3	0.94 ± 0.02	0.90 ± 0.02	1.63 ± 0.02	1.60 ± 0.02
22:6n-3	6.25 ± 0.54	5.50 ± 0.15	12.85 ± 0.28	12.64 ± 0.32
Σ SFA [#]	45.46 ± 0.67	47.04 ± 0.54	33.83 ± 0.29	34.39 ± 0.16
Σ MUFA [#]	6.80 ± 0.14	6.80 ± 0.06	6.30 ± 0.09	6.27 ± 0.14
Σ n-6 PUFA [#]	39.87 ± 0.55	39.34 ± 0.61	45.01 ± 0.52	44.72 ± 0.57
Σ n-3 PUFA [#]	7.88 ± 0.62	7.07 ± 0.16	14.86 ± 0.31	14.62 ± 0.34
n-6/n-3	5.25 ± 0.41	5.59 ± 0.21	3.04 ± 0.09	3.08 ± 0.11
(SFA+MUFA)/PUFA	1.10 ± 0.04	1.16 ± 0.02	0.67 ± 0.01	0.69 ± 0.01
Enzymes				
elongase	0.659 ± 0.023	0.647 ± 0.010	1.888 ± 0.029	1.814 ± 0.042
Δ5 desaturase	20.08 ± 1.60	19.90 ± 0.77	52.51 ± 1.23	48.76 ± 2.03
Δ6 desaturase	0.002 ± 0.0003	0.002 ± 0.0001	0.001 ± 0.000	0.001 ± 0.001
Δ9 desaturase	0.016 ± 0.0012	0.014 ± 0.0003	0.015 ± 0.001	0.014 ± 0.001

*[#] Σ SFA, Σ MUFA, Σ n-6 PUFA, Σ n-3 PUFA comprise of all fatty acids analyzed. Values are means ± SEM; spontaneously hypertensive rat (SHR), CD36 transgenic SHR (SHR-Cd36), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA); *P<0.05 vs. SHR.*

4.2.5 Dobutamine stress echocardiography

No significant differences were found in baseline systolic and diastolic dimensions of the LV between the two strains, except for slightly smaller LVD_S and larger PWT_S in SHR-Cd36 than in SHR (Table 5).

Table 5: Echocardiographic parameters of SHR and SHR-Cd36

Strain	AWT_d, mm	LVD_d, mm	PWT_d, mm	AWT_s, mm	LVD_s, mm	PWT_s, mm
SHR	1.87 ± 0.03	7.70 ± 0.13	1.78 ± 0.09	2.80 ± 0.07	4.81 ± 0.10	2.63 ± 0.11
SHR-Cd36	1.89 ± 0.05	7.58 ± 0.08	1.82 ± 0.03	2.95 ± 0.04	4.50 ± 0.06*	2.88 ± 0.06*

*Values are means ± SEM; spontaneously hypertensive rat (SHR), CD36 transgenic SHR (SHR-Cd36); diastolic anterior wall thickness (AWT_d), diastolic left ventricle diameter (LVD_d), diastolic posterior wall thickness (PWT_d), systolic anterior wall thickness (AWT_s), systolic left ventricle diameter (LVD_s), systolic posterior wall thickness (PWT_s); *P<0.05 vs. SHR.*

Assessment of systolic function revealed significantly higher fractional shortening in SHR-Cd36 as compared to SHR both at baseline and after the stimulation with increasing infusion rates of the β_1-AR agonist, dobutamine (Fig 14).

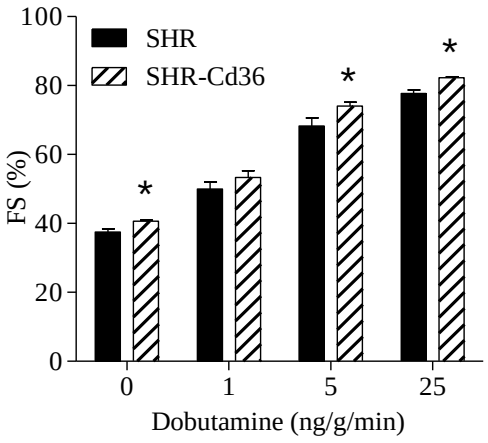

*Fig 14: Mean percent left ventricular fractional shortening (FS) measured in SHR and SHR-Cd36 at baseline and with increasing infusion rates of dobutamine. Values are means ± SEM, *P < 0.05 vs. SHR.*

4.2.6 β-Adrenoceptors

The total number of β-adrenergic binding sites in myocardial membrane preparations was determined by saturation binding with the β-AR agonist [^{3}H]CGP-12177 (Fig 15A). Analysis of the saturation binding curves indicated that myocardial membranes prepared from SHR-Cd36 had a significantly higher number β-ARs than those from SHR (28.6 ± 0.8 vs. 21.0 ± 2.0 fmol/mg protein) (Fig 15B). There was no difference in the binding affinity

of these receptors (K_D: 0.58 ± 0.11 vs. 0.68 ± 0.12 nM). Analysis of the competition binding isotherms revealed different proportions of β_1- and β_2-ARs subtypes in myocardial membranes from SHR and SHR-Cd36. Whereas in SHR the percentages of low- and high-affinity binding sites for ICI 118,551 were respectively 73 and 27 %, in SHR-Cd36 they were respectively 55 and 45 % (Fig 15C). In other words, the proportion of β_1- to β_2-ARs was 73 to 27 in SHR and 55 to 45 in SHR-Cd36. These data also indicate that the larger number of β-ARs in hearts of SHR-Cd36 was mainly caused by increased expression of β_2-ARs.

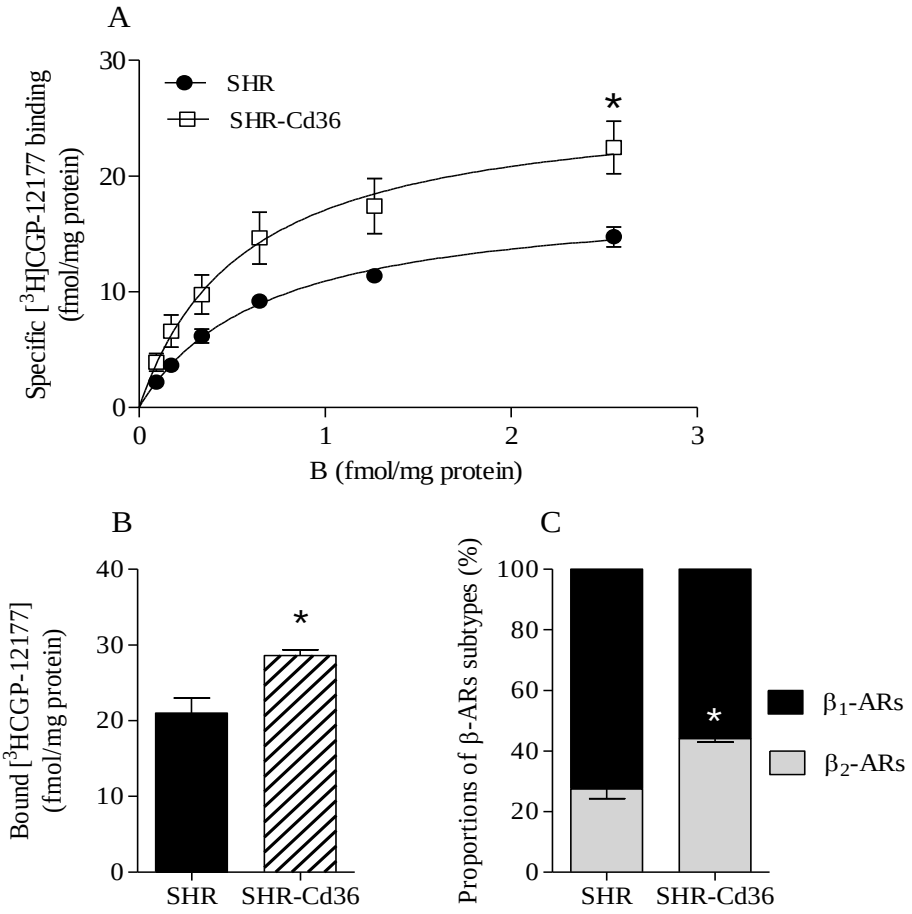

*Fig 15: Saturation binding curves showing specific binding of [3H]CGP-12177 to crude myocardial membranes of SHR (closed symbols) and SHR-Cd36 (open symbols) (A). Total number of β-ARs subtypes (B) and the relative proportion of β1- and β2-ARs (C) in myocardial preparations of SHR and SHR-Cd36. Values are means ± SEM; *P<0.05 vs. SHR.*

4.2.7 G proteins

No difference was observed in the protein content of $G_s\alpha$ between SHR-Cd36 and SHR hearts (Fig 16). Similarly, the protein content of myocardial $G_i\alpha$ was almost the same in both rat strains (Fig 16).

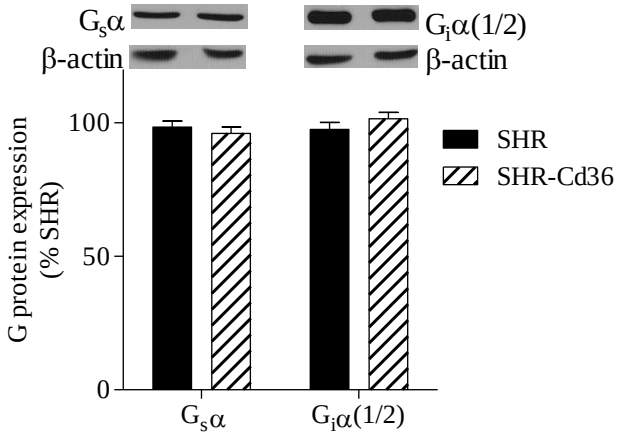

*Fig 16: Protein expression of $G_s\alpha$ and $G_i\alpha(1/2)$ in SHR and SHR-Cd36. β-Actin was used as a loading control. Values are means ± SEM; *P<0.05 vs. SHR.*

4.2.8 Adenylyl cyclase

Western blot analysis of the two dominant cardiac AC isoforms (AC5 and AC6) showed an increased amount of AC5/6 protein in SHR-Cd36 (by 20 %) when compared with SHR (Fig 17A).

To evaluate the functional status of the myocardial AC signaling system, the activity of AC was determined under basal conditions and after various stimulations (Fig 17B). Whereas the basal AC activity did not significantly differ between the two strains, the AC activity stimulated by GTPγS, isoproterenol (ISO), $MnCl_2$ and forskolin (FSK) was markedly increased (by 25 %, 18 %, 28 % and 30 %, respectively) in samples from SHR-Cd36 as compared to SHR.

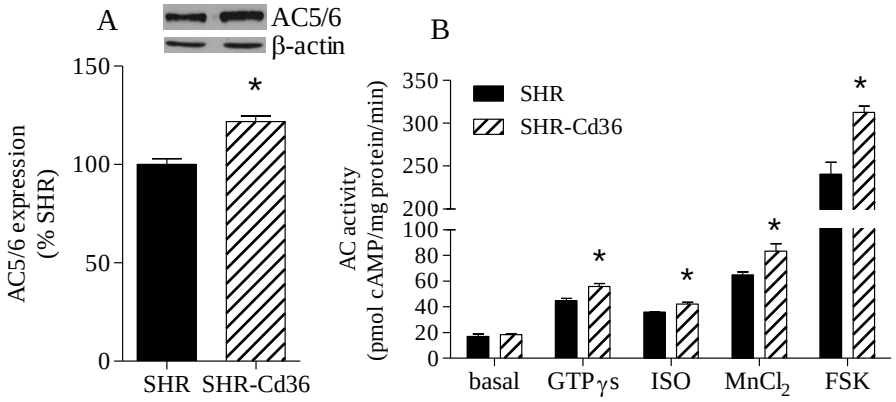

*Fig 17: Protein expression of AC5/6 in SHR and SHR-Cd36. β-Actin was used as a loading control (A). AC activity measured under basal conditions and after stimulation by GTPγS, isoproterenol (ISO), manganese ($MnCl_2$) or forskolin (FSK) (B). Values are means ± SEM; *P < 0.05 vs. SHR.*

4.2.9 Protein kinase A

Western blot analysis was performed to investigate the expression of PKA α and β C subunit and their Thr-198 phosphorylated form (p-PKA) in the LV of the two strains (Fig 18). The protein level of PKA and p-PKA, as well as the p-PKA/PKA ratio were higher (by 11%, 45% and 25%, respectively) in SHR-Cd36 than SHR, indicating increased activation of the enzyme in transgenic animals.

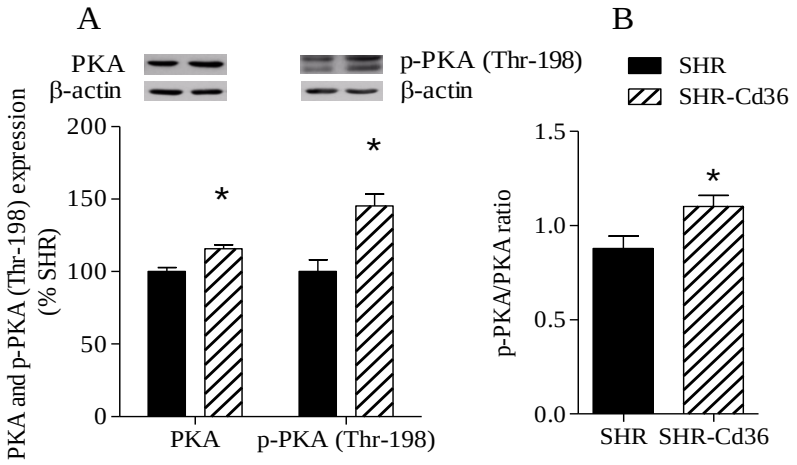

*Fig 19: Expression of protein kinase A catalytic subunit α and β and their phosphorylated forms (Thr-198) in the LV preparations of SHR and SHR-Cd36. β-Actin was used as a loading control (A). Bar graphs showing the ratios of p-PKA (Thr-198) to total PKA in myocardial preparations of SHR and SHR-Cd36 (B). Values are means ± SEM; *P<0.05 vs. SHR.*

5 DISCUSSION

5.1 AIM I

Within this aim, we studied the relationship between CD36-mediated FA transport, activation of two PKC isoforms and insulin resistance in the heart of the SHR and its congenic strain SHR-4 as well as the effect of a HSD feeding.

5.1.1 Hypertrophy, lipid and glucose metabolites and the effect of the HSD

In this study, we demonstrated that the congenic strain SHR-4 with a functional CD36 was less insulin-resistant, which is in line with previous findings (Pravenec *et al.*, 1999). Furthermore, we observed that myocardial LV hypertrophy, a characteristic feature of the SHR progenitor strain, was attenuated in the SHR-4 congenic strain (Klevstig *et al.*, 2011, supplement I). An earlier finding indicated that the cardiac hypertrophy in the SHR is accompanied with decreased FA β-oxidation and increased anaerobic metabolism (Labarthe *et al.*, 2005). A recent study showed that the hypertrophied SHR hearts exhibited alterations in the stroke volume, heart rate, late diastolic filling rate and increased aerobic glucose oxidation as a compensation for the suppressed β-oxidation (Dodd *et al.*, 2012), supporting the idea that a disruption in the balance of energy utilization can be involved in LV hypertrophy (Glatz *et al.*, 2006). We suppose that one of the factors leading to LV hypertrophy in the SHR is the lack of energy substrate due to the CD36 deficiency since the HSD suppressed the hypertrophy in the SHR. These findings are supported by studies, in which the diets containing either short-chain FA or medium-chain FA, that do not require CD36 facilitated transport, attenuated hypertrophy in the SHR (Hajri *et al.*, 2001; Shimojo *et al.*, 2004; Iemitsu *et al.*, 2008). Furthermore, CD36 have been proposed to be one of the major players in the development of heart hypertrophy (Pravenec *et al.*, 1995; Hajri *et al.*, 2001) as an imbalance of heart FA uptake and oxidation has been found in hypertrophied hearts (Bakermans *et al.*, 2011). The functional CD36 contributed also to lower serum insulin and FFA in the SHR-4 fed a standard chow and helped to prevent hyperglycemia and hyperinsulinemia after HSD feeding. Under the control condition, we

found a tendency to higher heart TAG concentration in the SHR-4 (Klevstig *et al.*, 2011, supplement I), which is in line with the finding showing an increased FA uptake in the SHR-4 in comparison with SHR (Hajri *et al.*, 2001). In addition, the insulin resistance stimulated by the HSD led to heart TAG accumulation in the SHR, which is one of the parameters of insulin resistance (Atkinson *et al.*, 2003). A study on isolated mitochondria showed that CD36 is located on the outer mitochondrial membrane and plays a role in FA oxidation (Smith *et al.*, 2011). It supports our hypothesis that the HSD activated the CD36 FA transport in SHR-4 not only into the cell but also into the mitochondrial compartment where the FA are oxidized. Thus, the heart TAG concentration in SHR-4 remained unchanged after the HSD feeding.

5.1.2 PKC δ and ε heart expression and the effect of HSD

The insertion of Cd36 into the SHR genom led to the improvement in insulin resistance, insulinemia, lipidemia and heart hypertrophy. This was surprisingly followed by increased PKC ε expression in the particulate fraction in the SHR-4. The increased membrane localization of PKC ε indicated its activation in the SHR-4 (Klevstig *et al.*, 2011, supplement I). Papers published so far have contradictorily shown that PKC ε activation plays a role in the development of insulin resistance in skeletal muscles (Laybutt *et al.*, 1999) by phosphorylating the transcription factor of the insulin receptor, High-mobility group AT-hook 1 (HMGA1). This leads to attenuation of the insulin receptor gene and protein expression and thus the insulin sensitivity is compromised (Dey *et al.*, 2007; Dasgupta *et al.*, 2011). On the other hand, when activated in the heart, PKC ε inhibits the hyperglycemia apoptosis signal, attenuates hyperglycemia-induced oxidative stress and thereby prevents diabetic cardiomyopathy (Malhotra *et al.*, 2005). PKC ε plays also a role in the compensatory phase of hypertrophic growth (Wu *et al.*, 2000; Inagaki *et al.*, 2008) and/or in the cardioprotection (Rafiee *et al.*, 2002; Lange *et al.*, 2007). On the basis of our results, we presume that the activated PKC ε in SHR-4 is connected with the inserted Cd36 and the improved LCFA transport to the heart (Hajri *et al.*, 2001), as it is known that LCFA can activate PKC (Huang *et al.*, 1997; Ragheb *et al.*, 2009).

HSD feeding had different effects on the two novel PKC isoforms in the two strains. The PKC δ was upregulated and PKC ε downregulated in the heart of SHR-4 after the HSD together with increased insulin serum concentration, while no effect was observed in

the SHR (Klevstig *et al.*, 2011, supplement I). The disorder in the susceptibility of the PKC isoforms to HSD load seen in the SHR is in line with the results of Labarthe *et al.* (2005), who observed an impaired capacity to respond and tolerate an acute stress, induced by 30 min epinephrine stimulation, in the perfused SHR heart compared to the normotensive Wistar rat. They also proved that impaired LCFA metabolism as a result of CD36 absence is a key factor determining the heart resistance to stress (Labarthe *et al.*, 2005). Insulin is known to activate PKC δ in skeletal muscle, where it can act down from the insulin receptor in the insulin signaling pathway (Jacob *et al.*, 2010) or terminate the insulin signal by feedback inhibition (Waraich *et al.*, 2008). Similarly to PKC δ, PKC ε is also known to be a modulator of insulin signaling; it can inhibit the insulin pathway in skeletal muscle when activated by glucose infusion (Laybutt *et al.*, 1999).

It seems, however, that the response of both PKC δ and PKC ε to insulin and glucose infusion in the heart differs from those in skeletal muscle. Luiken *et al.* (2009) observed no change in PKC δ and/or PKC ε expression or translocation after acute insulin administration in isolated cardiac myocytes from Lewis rats. Moreover, D'Alessandro *et al.* (2008) showed increased expression of PKC ε in the particulate fraction in the heart of HSD-induced insulin-resistant rats. It should be pointed out, that there were no changes in the insulin concentration between the rats fed the HSD and the controls. The aforementioned findings lead us to the assumption that the PKC isoforms in our study could also be regulated by other factors than the elevated insulin concentration. HSD feeding represents a metabolic stress and is accompanied by enhanced production of ROS (Busserolles *et al.*, 2002). We suppose that our results present a possible link between the function of CD36 and PKC δ activation in reaction to the HSD load in the genetically modified SHR-4. This assumption is supported by the study in which stress induced by adaptation to chronic hypoxia in Wistar rats led to similar shift in PKC δ (Kolar *et al.*, 2007; Hlavackova *et al.*, 2010) as occurred after the HSD load in the SHR-4. In the chronic hypoxia study, the PKC δ upregulation was ROS-dependent and cardioprotective. We confirmed that the improved insulin sensitivity in SHR-4 rats was associated with the CD36 and increased PKC ε expression. Moreover, we showed that the SHR-4 heart was able to respond to the HSD load by regulating both the PKC ε and PKC δ expressions. However, further investigations are needed to resolve whether the two PKC isoforms are

directly involved in the improved insulin sensitivity in the SHR-4 and if they participate in signaling pathway leading to CD36 translocation.

5.2 AIM II

After analyzing the effect of CD36 on PKC expression and insulin resistance, we wanted to examine its role in the tolerance to I/R injury. First we compared the incidence and severity of I/R ventricular arrhythmias and MI induced by coronary occlusion in the SHR-4 congenic strain and SHR. Since there were no significant differences in arrhythmias and IS between the two strains, we decided that the study would be performed on the trangenic SHR-Cd36 with a wild-type Cd36 in comparison with progenitor SHR. The higher CD36 expression in the transgenic SHR-CD36 strain when compared to the SHR-4 (Pravenec *et al.*, 2008a) could explain the discrepancies in the tolerance to I/R injury between the SHR-4 and SHR-Cd36.

5.2.1 Effect of CD36 on I/R injury

It has been suggested that increased uptake of FA by the heart after MI might represent a metabolic cause leading to fatal ventricular fibrillation during acute myocardial ischemia (Oliver, 2001). It is well known that CD36 contributes markedly to the regulation of FA uptake, oxidation and esterification in the heart as more than 50% of LCFA uptake by the heart occurs via sarcolemmal CD36-mediated transport (Kuang *et al.*, 2004; Luiken *et al.*, 2004). In the current study, we found that transgenic expression of Cd36 in the SHR was associated with markedly increased susceptibility to ventricular arrhythmias induced by I/R and reduced myocardial IS (Neckar *et al.*, 2012, supplement II). Accordingly, it is possible that transgenic SHR with wild-type Cd36 are predisposed to arrhythmias due to increased FA uptake. The mechanisms of these FA-associated proarrhythmogenic effects are not fully understood but they might include an increased requirement of oxygen for FA catabolism (Oliver, 2006; Lam & Lopaschuk, 2007), an accumulation of potentially toxic intermediates of FA metabolism such as long-chain acylcarnitine and long-chain acyl coenzyme A, or a FA-mediated inhibition of glucose utilization by myocardium (Bonnet *et al.*, 1999; Oliver, 2006; Turer *et al.*, 2009). On the other hand, SHR rats with mutant Cd36, with reduced FA transport and oxidation in the heart and with increased glucose oxidation

for ATP production are relatively protected from adverse metabolic effects of increased FA levels (Neckar *et al.*, 2012, supplement II).

To our surprise, isolated hearts of SHR-Cd36 perfused with a crystalloid solution free of FA were also more susceptible to ischemic ventricular arrhythmias compared to SHR (Neckar *et al.*, 2012, supplement II). These findings suggest that the proarrhythmic effect in SHR-Cd36 is independent of FA uptake and is an intrinsic myocardial property. Furthermore, catecholamine depletion by reserpine pretreatment eliminated the increased amount of arrhythmias in the hearts of SHR-Cd36, indicating an important role of catecholamines in triggering the ischemia-induced arrhythmias in this strain. Indeed, previous findings showed an impaired myocardial contractility as a respond to α-AR as well as β-AR stimulation in the SHR when compared to control rats (Labarthe *et al.*, 2005). In addition, Cd36 was originally identified as a quantitative trait gene regulating β-AR stimulation induced lipolysis in isolated adipocytes when the SHR mutant allele was associated with reduced sensitivity to catecholamines (Aitman *et al.*, 1997). Contrary to increased arrhythmogenesis in the transgenic SHR-Cd36, the expression of wild-type Cd36 was associated with significantly reduced myocardial IS determined both in open-chest rats and isolated perfused hearts (Neckar *et al.*, 2012, supplement II). Opposite changes of the two endpoints of ischemia can be explained by different factors that are involved in the pathogenic mechanisms. In line with our results Irie *et al.* (2003) found that CD36-null mice were energetically deficient and more sensitive to acute ischemic insult, similarly to the SHR. However, the impaired cardiac ischemic tolerance was not confirmed in another study using the same mouse model (Kuang *et al.*, 2004). Moreover, the inhibition of CD36 exerted reduced IS after I/R injury (Bessi *et al.*, 2012). Whereas CD36 inhibition prevented the loss of cardiac function in a model of lipid-induced cardiac insulin resistance (Angin *et al.*, 2012), the cardiac performance in Cd36 knockout mice were depressed (Pietka *et al.*, 2012). In view of the limited data available, it is currently impossible to explain these contradictory results. Taken together, the description up to date of the CD36 role on cardiac function as well as tolerance to I/R injury have been contradictory,

Membrane fluidity changes due to altered FA composition of membrane phospholipids have been proven to play a role in the protection against I/R injury (Pepe & McLennan, 2002; Hlavackova *et al.*, 2007; Zeghichi-Hamri *et al.*, 2010). It has been widely accepted that n-3 PUFA, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA),

incorporation into myocardial membrane phospholipids have a protective effect to I/R insult (Zeghichi-Hamri *et al.*, 2010; Rauch & Senges, 2012). Furthermore, the FA composition of myocardial membrane phospholipid is sensitive to the type of FA consumed in the diet (Hlavackova *et al.*, 2007; Balkova *et al.*, 2009; Slee *et al.*, 2010). Therefore, one would expect an increased incorporation of LCFA into the myocardial membrane phospholipids in the SHR-Cd36, but no differences were observed. Accordingly, we can presume that the increased amount of FA taken up by the SHR-Cd36 myocardium is used for energy production, as a higher respiration rate and lower TAG accumulation was observed in the SHR-Cd36 when compared to the SHR (Neckar *et al.*, 2012, supplement II).

5.2.2 β-AR signaling pathway in the proarrhythmogenic effect of CD36

It is well documented that increased β-adrenergic sensitivity and/or overexpression of β-ARs leads to cardiac hypertrophy (Engelhardt *et al.*, 1999; Liggett *et al.*, 2000). Here we found that the transgenic SHR-Cd36 strain with increased β-AR expression had higher heart and LV weights when compared with SHR (Klevstig *et al.*, in press, supplement III). These results are in agreement with the findings showing that CD36 overexpression in mice caused cardiac hypertrophy (Sung *et al.*, 2011). On the other hand, we and other investigators reported that CD36 mutation may lead to cardiac hypertrophy (Pravenec *et al.*, 1995; Hall *et al.*, 2011; Klevstig *et al.*, 2011, supplement I). Hence, these observations suggest that any distinction in the CD36 function, mutation or overexpression, could result in cardiac hypertrophy.

Signaling mediated by the β-AR/AC pathway is known to play a fundamental role in modulating cardiomyocyte contractility (Lissandron & Zaccolo, 2006) and increased β-AR stimulation has been shown to contribute to fatal arrhythmias (Bassiakou *et al.*, 2009). On the other hand, β-ARs have also been proposed to play an important role in the recovery after ischemic injury, as the protective effect of ischemic precondition was abrogated after β-AR blocking (Penson *et al.*, 2008). Previous findings have also shown that a modest stimulation of β-ARs before ischemic injury, so call β-adrenergic preconditioning, reduced the prevalence of arrhythmias and improved the functional recovery after ischemia (Maier *et al.*, 2009). Taken together, depending on the strength of

the stimulation, the β-AR signaling pathway contributes both to the damage as well as the protection of ischemic injury. The observations on isoproterenol-stimulated β-ARs in isolated cardiomyocytes from β_2-AR but not β_1-AR knockout mice led to contractile response of myocytes through an increased cAMP production, sustained PKA activity and PKA phosphorylation of phospholamban (Soto *et al.*, 2009). These data indicate that the contraction response is primarily due to β_1-AR stimulation. Importantly, myocardial IS and arrhythmias induced by coronary artery ligation can be reduced by the blockade of β_1-ARs, which results in a diminution of intracellular cAMP levels and PKA activation (Lu *et al.*, 2009; Zhang *et al.*, 2010). The increased myocardial β-AR density and responsiveness in SHR-Cd36 when compared to SHR (Klevstig *et al.*, in press, supplement III) may thus well explain major increase in arrhythmogenesis observed in the transgenic animals (Neckar *et al.*, 2012, supplement II). It is important to note that especially β_2-ARs were up-regulated in the hearts of SHR-Cd36 (Klevstig *et al.*, in press, supplement III). There is accumulating evidence that β_2-ARs can mediate anti-apoptotic signaling through activation of G_i, PI3K and Akt pathway (Zhu *et al.*, 2001; Bernstein *et al.*, 2011). Hence, the higher number of these receptors could account for the lower myocardial IS after coronary artery occlusion in SHR-Cd36 (Neckar *et al.*, 2012, supplement II). Nevertheless, more research is needed to confirm this assumption.

Trimeric G proteins function as key regulatory elements in signal transduction from activated β-ARs and they can be altered under different pathophysiological conditions, such as cardiac hypertrophy, arrhythmia and heart failure (Zolk *et al.*, 2000; Slotkin *et al.*, 2003). Therefore, it was of interest to investigate the expression of selected G protein subunits in the heart of SHR-Cd36 and SHR rats. We did not find any difference in the amount of α subunits of the G_s and G_i proteins in myocardial membrane preparation from both rat strains (Klevstig *et al.*, in press, supplement III). Although there were no appreciable changes in G protein expression, differently regulated AC activity was higher in myocardial membranes from SHR-Cd36 than SHR. A significantly increased AC activity was determined after direct stimulation of the enzyme by Mn^{2+} ions or after stimulation of both the enzyme and G_s protein by forskolin, as well as after stimulation of G_s protein by GTPγS or after stimulation of β-ARs by isoproterenol. The enhanced sensitivity of the enzyme to stimulation may be explained by higher protein expression of AC5/6 in myocardial preparations from SHR-Cd36 (Klevstig *et al.*, in press, supplement

III). Both AC5 and AC6 have been shown to play an important role in the contractile response of myocardium. Okumura *et al.* (2007) observed a reduction of LV ejection fraction response to β-AR stimulation in AC5 knock-out mice and Tang *et al.* (2008) found that AC6 deletion impaired β-AR-stimulated cAMP production and led to diminished contractility responsiveness upon stimulation with dobutamine. On the other hand, mice overexpressing AC6 displayed an increased contractile response to dobutamine (Takahashi *et al.*, 2006). In line with these studies, we found significantly increased responsiveness of the LV systolic function to dobutamine in SHR-Cd36 with higher AC expression and activity as compared to SHR (Klevstig *et al.*, in press, supplement III).

PKA phosphorylates and activates a number of key excitation-contraction coupling proteins, such as PLB, LTCC, RyR and TnI (Xiang & Kobilka, 2003). It has previously been shown that β-AR stimulation leads to PKA-mediated phorphorylation of PLB and TnI (Di Benedetto *et al.*, 2008) and PKA C subunit overexpression results in dilated cardiomyopathy and arrhythmias (Antos *et al.*, 2001). Our present results are in agreement with these observations. SHR-Cd36 with increased arrhythmias and contractility response have increased myocardial expression of the PKA C subunit, phosphorylated PKA C subunit Thr-198 as well as the p-PKA/PKA ratio, which indicates increased PKA activity (Klevstig *et al.*, in press, supplement III).

To date, no clear link between the function of CD36 and β-AR signaling in the heart has been reported. Nevertheless, some earlier studies indicated that SHR express a lower number of cardiac membrane-bound β-ARs as compared to normotensive WKY rats and that myocardial AC activity is lower in the former than in the latter strain (Anandsrivastava, 1988; Matsumori *et al.*, 1989). On the other hand, a recent finding by Pietka *et al.* (2012) showed higher cAMP levels and increased PKA activity in cardiomyocytes of a Cd36 knockout mouse when compared to its littermates. Our present findings provide strong evidence that reintroduction of the wild-type Cd36 gene into the genome of SHR may reverse the diminished myocardial AC activity in the SHR. The upregulation of some components of the β-AR-mediated AC signaling system observed in SHR-Cd36 transgenic rats was accompanied by increased cardiac β-AR responsiveness, sensitization of AC and activation of PKA, which led to a higher susceptibility of these animals to ischemia-induced arrhythmias. Further research will be required to elucidate which molecular mechanisms underlie these effects of the Cd36 gene.

6 SUMMARY

The main aim of this work was to investigate the effect of wild-type Cd36 on cardiac function and on different signaling pathways in the SHR and the genetically modified strains, SHR-4 and SHR-Cd36.

AIM I

- We demonstrated that insertion of a segment of chromosome 4, which contains the Cd36 gene, from the BN strain into SHR genome lowered the serum FFA, insulin and glucose concentrations, indicating improved insulin sensitivity in the SHR-4 congenic strain.

- The SHR-4 exhibited higher PKC ε expression and its increased translocation to membrane fraction, suggesting elevated PKC ε activation. This is supporting the proposal that the SHR-4 strain have improved LCFA uptake in comparison with the SHR, which can activate this PKC isoform.

- We found different response of the two strains to the HSD feeding. Whereas the HSD impaired the lipid and glucose metabolic traits in the SHR, the SHR-4 tolerated the sucrose overload and did not exhibit any signs of insulin resistance. Furthermore, the PKC ε and PKC δ isoforms were affected by the HSD feeding only in the SHR-4. These results show that the Cd36 gene affects the ability to respond to a sucrose overload by regulating the PKC δ and PKC ε expression.

AIM II

- Using the transgenic SHR-Cd36 strain, with a wild-type Cd36, we showed that the insertion of the Cd36 gene affects ischemic tolerance. We found that SHR-Cd36 had increased incidence and severity of ischemic ventricular arrhythmias and smaller myocardial IS induced by coronary artery occlusion.

- We did not observe any significant difference in the FA composition of heart and serum phospholipids between the strains, indicating that the increased arrythmogenesis in the SHR-Cd36 is not due to altered FA composition of heart phospholipids, which are known to be involved in the tolerance against I/R injury.

- Reserpine abrogated the increased incidence and severity of ischemic ventricular arrhythmias in the SHR-Cd36, suggesting the involvement of catecholamines. The dobutamine stress test intensified this hypothesis, as we observed an increased heart response to β-AR stimulation in the transgenic strain.

- We demonstrated that the presence of wild-type Cd36 upregulated some components of the β-AR signaling pathway. We observed increased β-AR density, AC responsiveness and higher protein expression of AC and PKA. These findings indicate a connection between the Cd36 gene, β-AR signaling pathway and susceptibility to I/R arrhythmias in the SHR.

It can be concluded that the CD36 function plays an important role in the heart energy metabolism through the regulation of the PKC δ and PKC ε isoforms. Furthermore, CD36 also modulates the tolerance to I/R injury in a β-AR signaling pathway-dependent manner. Taken together, the function of CD36 affects various pathophysiological mechanisms and signaling pathways in the heart and is an important component in maintaining normal physiological cardiovascular functions.

7 References

Aitman TJ, Glazier AM, Wallace CA, Cooper LD, Norsworthy PJ, Wahid FN, Al-Majali KM, Trembling PM, Mann CJ, Shoulders CC, Graf D, St Lezin E, Kurtz TW, Kren V, Pravenec M, Ibrahimi A, Abumrad NA, Stanton LW & Scott J (1999). Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. *Nat Genet* **21,** 76-83.

Aitman TJ, Gotoda T, Evans AL, Imrie H, Heath KE, Trembling PM, Truman H, Wallace CA, Rahman A, Dore C, Flint J, Kren V, Zidek V, Kurtz TW, Pravenec M & Scott J (1997). Quantitative trait loci for cellular defects in glucose and fatty acid metabolism in hypertensive rats. *Nat Genet* **16,** 197-201.

Akazawa H & Komuro I (2003). Roles of cardiac transcription factors in cardiac hypertrophy. *Circ Res* **92,** 1079-1088.

Anandsrivastava MB (1988). Altered responsiveness of adenylate-cyclase to adenosine and other agents in the myocardial sarcolemma and aorta of spontaneously-hypertensive rats. *Biochem Pharmacol* **37,** 3017-3022.

Angin Y, Steinbusch LK, Simons PJ, Greulich S, Hoebers NT, Douma K, van Zandvoort MA, Coumans WA, Wijnen W, Diamant M, Ouwens DM, Glatz JF & Luiken JJ (2012). CD36 inhibition prevents lipid accumulation and contractile dysfunction in rat cardiomyocytes. *Biochem J* **448,** 43-53.

Antos CL, Frey N, Marx SO, Reiken S, Gaburjakova M, Richardson JA, Marks AR & Olson EN (2001). Dilated cardiomyopathy and sudden death resulting from constitutive activation of protein kinase A. *Circ Res* **89,** 997-1004.

Ashraf MZ & Gupta N (2011). Scavenger receptors: implications in atherothrombotic disorders. *Int J Biochem Cell B* **43,** 697-700.

Atkinson LL, Kozak R, Kelly SE, Onay-Besikci A, Russell JC & Lopaschuk GD (2003). Potential mechanisms and consequences of cardiac triacylglycerol accumulation in insulin-resistant rats. *Am J Physiol-Endoc M* **284,** E923-E930.

Bakermans AJ, Geraedts TR, van Weeghel M, Denis S, Ferraz MJ, Aerts JMFG, Aten J, Nicolay K, Houten SM & Prompers JJ (2011). Fasting-induced myocardial lipid accumulation in long-chain acyl-CoA dehydrogenase knockout mice is accompanied by impaired left ventricular function. *Circ-Cardiovasc Imag* **4,** 558-565.

Balkova P, Jezkova J, Hlavackova M, Neckar J, Stankova B, Kolar F, Novak F & Novakova O (2009). Dietary polyunsaturated fatty acids and adaptation to chronic hypoxia alter acyl composition of serum and heart lipids. *Brit J Nutr* **102,** 1297-1307.

Bassiakou E, Xanthos T & Papadimitriou L (2009). The potential beneficial effects of beta adrenergic blockade in the treatment of ventricular fibrillation. *Eur J Pharmacol* **616,** 1-6.

Beazely MA & Watts VJ (2006). Regulatory properties of adenylate cyclases type 5 and 6: a progress report. *Eur J Pharmacol* **535,** 1-12.

Bernstein D, Fajardo G & Zhao M (2011). The role of beta-adrenergic receptors in heart

failure: differential regulation of cardiotoxicity and cardioprotection. *Prog Pediatr Cardiol* **31,** 35-38.

Bessi VL, Labbe SM, Huynh DN, Menard L, Jossart C, Febbraio M, Guerin B, Bentourkia M, Lecomte R, Carpentier AC, Ong H & Marleau S (2012). EP 80317, a selective CD36 ligand, shows cardioprotective effects against post-ischaemic myocardial damage in mice. *Cardiovasc Res* **96,** 99-108.

Bezaire V, Bruce CR, Heigenhauser GJF, Tandon NN, Glatz JFC, Luiken JJJF, Bonen A & Spriet LL (2006). Identification of fatty acid translocase on human skeletal muscle mitochondrial membranes: essential role in fatty acid oxidation. *Am J Physiol-Endoc M* **290,** E509-E515.

Bhattacharya S, Dey D & Roy SS (2007). Molecular mechanism of insulin resistance. *J Biosciences* **32,** 405-413.

Bing OHL, Brooks WW, Robinson KG, Slawsky MT, Hayes JA, Litwin SE, Sen S & Conrad CH (1995). The spontaneously hypertensive rat as a model of the transition from compensated left-ventricular hypertrophy to failure. *J Mol Cell Cardiol* **27,** 383-396.

Bonen A, Han XX, Tandon NN, Glatz JF, Lally J, Snook LA & Luiken JJ (2009). FAT/CD36 expression is not ablated in spontaneously hypertensive rats. *J Lipid Res* **50,** 740-748.

Bonnet D, Martin D, de Lonlay P, Villain E, Jouvet P, Rabier D, Brivet M & Saudubray JM (1999). Arrhythmias and conduction defects as presenting symptoms of fatty acid oxidation disorders in children. *Circulation* **100,** 2248-2253.

Bradford MM (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal Biochem* **72,** 248-254.

Brodde OE, Bruck H & Leineweber K (2006). Cardiac adrenoceptors: Physiological and pathophysiological relevance. *J Pharmacol Sci* **100,** 323-337.

Busserolles J, Zimowska W, Rock E, Rayssiguier Y & Mazur A (2002). Rats fed a high sucrose diet have altered heart antioxidant enzyme activity and gene expression. *Life Sci* **71,** 1303-1312.

Chabowski A, Gorski J, Calles-Escandon J, Tandon NN & Bonen A (2006). Hypoxia-induced fatty acid transporter translocation increases fatty acid transport and contributes to lipid accumulation in the heart. *Febs Lett* **580,** 3617-3623.

Chen HC, Bandyopadhyay G, Sajan MP, Kanoh Y, Standaert M, Farese RV & Farese RV (2002). Activation of the ERK pathway and atypical protein kinase C Isoforms in exercise- and aminoimidazole-4-carboxamide-1-beta-D-riboside (AICAR)-stimulated glucose transport. *J Biol Chem* **277,** 23554-23562.

Chen L, Hahn H, Wu GY, Chen CH, Liron T, Schechtman D, Cavallaro G, Banci L, Guo YR, Bolli R, Dorn GW & Mochly-Rosen D (2001). Opposing cardioprotective actions and parallel hypertrophic effects of delta PKC and epsilon PKC. *P Natl Acad Sci USA* **98,** 11114-11119.

Cheng XD, Ji ZY, Tsalkova T & Mei F (2008). Epac and PKA: a tale of two intracellular cAMP receptors. *Acta Bioch Bioph Sin* **40,** 651-662.

Chess DJ & Stanley WC (2008). Role of diet and fuel overabundance in the development and progression of heart failure. *Cardiovasc Res* **79,** 269-278.

Cipolletta E, Campanile A, Santulli G, Sanzari E, Leosco D, Campiglia P, Trimarco B & Iaccarino G (2009). The G protein coupled receptor kinase 2 plays an essential role in beta-adrenergic receptor-induced insulin resistance. *Cardiovasc Res* **84,** 407-415.

Coburn CT, Knapp FF, Febbraio M, Beets AL, Silverstein RL & Abumrad NA (2000). Defective uptake and utilization of long chain fatty acids in muscle and adipose tissues of CD36 knockout mice. *J Biol Chem* **275,** 32523-32529.

Coort SLM, Bonen A, van der Vusse GJ, Glatz JFC, Luiken JJFP (2007) Cardiac substrate uptake and metabolism in obesity and type-2 diabetes: Role of sarcolemmal substrate transporters. *Mol Cell Biochem* **299**, 5-18.

Coort SLM, Hasselbaink DM, Koonen DPY, Willems J, Coumans WA, Chabowski A, van der Vusse GJ, Bonen A, Glatz JFC & Luiken JJFP (2004). Enhanced sarcolemmal FAT/CD36 content and triacylglycerol storage in cardiac myocytes from obese Zucker rats. *Diabetes* **53,** 1655-1663.

Corpeleijn E, van der Kallen CJH, Kruijshoop M, Magagnin MGP, de Bruin TWA, Feskens EJM, Saris WHM & Blaak EE (2006). Direct association of a promoter polymorphism in the CD36/FAT fatty acid transporter gene with Type 2 diabetes mellitus and insulin resistance. *Diabetic Med* **23,** 907-911.

D'Alessandro ME, Chicco A & Lombardo YB (2008). Dietary fish oil reverses lipotoxicity, altered glucose metabolism, and nPKC epsilon translocation in the heart of dyslipemic insulin-resistant rats. *Metabolism* **57,** 911-919.

Dai WD, Simkhovich BZ & Kloner RA (2009). Ischemic preconditioning maintains cardioprotection in aging normotensive and spontaneously hypertensive rats. *Exp Gerontol* **44,** 344-349.

Dasgupta S, Bhattacharya S, Maitra S, Pal D, Majumdar SS, Datta A & Bhattacharya S (2011). Mechanism of lipid induced insulin resistance: activated PKC epsilon is a key regulator. *Bba-Mol Basis Dis* **1812,** 495-506.

Davidoff AJ, Davidson MB, Carmody MW, Davis ME & Ren J (2004). Diabetic cardiomyocyte dysfunction and myocyte insulin resistance: role of glucose-induced PKC activity. *Mol Cell Biochem* **262,** 155-163.

Defer N, Best-Belpomme M & Hanoune J (2000). Tissue specificity and physiological relevance of various isoforms of adenylyl cyclase. *Am J Physiol-Renal* **279,** F400-F416.

DeFronzo RA (2010). Insulin resistance, lipotoxicity, type 2 diabetes and atherosclerosis: the missing links. The Claude Bernard Lecture 2009. *Diabetologia* **53,** 1270-1287.

Dey D, Bhattacharya A, Roy S & Bhattacharya S (2007). Fatty acid represses insulin receptor gene expression by impairing HMGA1 through protein kinase C epsilon. *Biochem Bioph Res Co* **357,** 474-479.

Di Benedetto G, Zoccarato A, Lissandron V, Terrin A, Li X, Houslay MD, Baillie GS & Zaccolo M (2008). Protein kinase A type I and type II define distinct intracellular signaling compartments. *Circ Res* **103,** 836-844.

Dirkx E, Schwenk RW, Glatz JFC, Luiken JJFP & van Eys GJJM (2011). High fat diet induced diabetic cardiomyopathy. *Prostag Leukotr Ess* **85,** 219-225.

Dodd MS, Ball DR, Schroeder MA, Le Page LM, Atherton HJ, Heather LC, Seymour AM, Ashrafian H, Watkins H, Clarke K & Tyler DJ (2012). In vivo alterations in cardiac metabolism and function in the spontaneously hypertensive rat heart. *Cardiovasc Res* **95,** 69-76.

Duquesnes N, Lezoualc'h F & Crozatier B (2011). PKC-delta and PKC-epsilon: foes of the same family or strangers? *J Mol Cell Cardiol* **51,** 665-673.

Edwards HV, Christian F & Baillie GS (2012). cAMP: Novel concepts in compartmentalised signalling. *Semin Cell Dev Biol* **23,** 181-190.

El-Armouche A & Eschenhagen T (2009). beta-Adrenergic stimulation and myocardial function in the failing heart. *Heart Fail Rev* **14,** 225-241.

Engelhardt S, Hein L, Wiesmann F & Lohse MJ (1999). Progressive hypertrophy and heart failure in beta(1)-adrenergic receptor transgenic mice. *P Natl Acad Sci USA* **96,** 7059-7064.

Falcao-Pires I & Leite-Moreira AF (2012). Diabetic cardiomyopathy: understanding the molecular and cellular basis to progress in diagnosis and treatment. *Heart Fail Rev* **17,** 325-344.

Febbraio M, Abumrad NA, Hajjar DP, Sharma K, Cheng WL, Pearce SFA & Silverstein RL (1999). A null mutation in murine CD36 reveals an important role in fatty acid and lipoprotein metabolism. *J Biol Chem* **274,** 19055-19062.

Febbraio M & Silverstein RL (2007). CD36: Implications in cardiovascular disease. *Int J Biochem Cell B* **39,** 2012-2030.

Fliegner D, Westermann D, Riad A, Schubert C, Becher E, Fielitz J, Tschope C & Regitz-Zagrosek V (2008). Up-regulation of PPAR gamma in myocardial infarction. *Eur J Heart Fail* **10,** 30-38.

Folch J, Lees M & Stanley GHS (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226,** 497-509.

Frangioudakis G, Burchfield JG, Narasimhan S, Cooney GJ, Leitges M, Biden TJ & Schmitz-Peiffer C (2009). Diverse roles for protein kinase C delta and protein kinase C epsilon in the generation of high-fat-diet-induced glucose intolerance in mice: regulation of lipogenesis by protein kinase C delta. *Diabetologia* **52,** 2616-2620.

Gauthier C, Seze-Goismier C & Rozec B (2007). Beta 3-adrenoceptors in the cardiovascular system. *Clin Hemorheol Micro* **37,** 193-204.

Glatz JF, Bonen A, Ouwens DM & Luiken JJ (2006). Regulation of sarcolemmal transport of substrates in the healthy and diseased heart. *Cardiovasc Drugs Ther* **20,** 471-476.

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

Gotoda T, Iizuka Y, Kato N, Osuga J, Bihoreau MT, Murakami T, Yamori Y, Shimano H, Ishibashi S & Yamada N (1999). Absence of Cd36 mutation in the original spontaneously hypertensive rats with insulin resistance. *Nat Genet* **22,** 226-228.

Goudriaan JR, Dahlmans VEH, Teusink B, Ouwens DM, Febbraio M, Maassen JA, Romijn JA, Havekes LM & Voshol PJ (2003). CD36 deficiency increases insulin sensitivity in muscle, but induces insulin resistance in the liver in mice. *J Lipid Res* **44,** 2270-2277.

Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC, Spertus JA & Costa F (2005). Diagnosis and management of the metabolic syndrome - An American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation* **112,** 2735-2752.

Habets DDJ, Coumans WA, El Hasnaoui M, Zarrinpashneh E, Bertrand L, Viollet B, Kiens B, Jensen TE, Richter EA, Bonen A, Glatz JFC & Luiken JJFP (2009). Crucial role for LKB1 to AMPK alpha 2 axis in the regulation of CD36-mediated long-chain fatty acid uptake into cardiomyocytes. *Bba-Mol Cell Biol L* **1791,** 212-219.

Hajri T, Han XX, Bonen A & Abumrad NA (2002). Defective fatty acid uptake modulates insulin responsiveness and metabolic responses to diet in CD36-null mice. *J Clin Invest* **109,** 1381-1389.

Hajri T, Ibrahimi A, Coburn CT, Knapp FF, Kurtz T, Pravenec M & Abumrad NA (2001). Defective fatty acid uptake in the spontaneously hypertensive rat is a primary determinant of altered glucose metabolism, hyperinsulinemia, and myocardial hypertrophy. *J Biol Chem* **276,** 23661-23666.

Hall D, Mayosi BM, Rahman TJ, Avery PJ, Watkins HC & Keavney B (2011). Common variation in the CD36 (fatty acid translocase) gene is associated with left-ventricular mass. *J Hypertens* **29,** 690-695.

Harmon CM & Abumrad NA (1993). Binding of sulfosuccinimidyl fatty-acids to adipocyte membrane-proteins - Isolation and amino-terminal sequence of an 88-kD protein implicated in transport of long-chain fatty-acids. *J Membrane Biol* **133,** 43-49.

Hawley SA, Pan DA, Mustard KJ, Ross L, Bain J, Edelman AM, Frenguelli BG & Hardie DG (2005). Calmodulin-dependent protein kinase kinase-beta is an alternative upstream kinase for AMP-activated protein kinase. *Cell Metab* **2,** 9-19.

Heather LC, Cole MA, Lygate CA, Evans RD, Stuckey DJ, Murray AJ, Neubauer S & Clarke K (2006). Fatty acid transporter levels and palmitate oxidation rate correlate with ejection fraction in the infarcted rat heart. *Cardiovasc Res* **72,** 430-437.

Hlavackova M, Kozichova K, Neckar J, Kolar F, Musters RJP, Novak F & Novakova O (2010). Up-regulation and redistribution of protein kinase C-delta in chronically hypoxic heart. *Mol Cell Biochem* **345,** 271-282.

Hlavackova M, Neckar J, Jezkova J, Balkova P, Stankova B, Novakova O, Kolar F & Novak F (2007). Dietary polyunsaturated fatty acids alter myocardial protein kinase C expression and affect cardioprotection induced by chronic hypoxia. *Exp Biol Med* **232,** 823-832.

Ho D, Yan L, Iwatsubo K, Vatner DE & Vatner SF (2010). Modulation of beta-adrenergic receptor signaling in heart failure and longevity: targeting adenylyl cyclase type 5. *Heart Fail Rev* **15,** 495-512.

Hoosdally SJ, Andress EJ, Wooding C, Martin CA & Linton KJ (2009). The human scavenger receptor CD36 glycosylation status and its role in trafficking and function. *J Biol Chem* **284,** 16277-16288.

Huang XP, Pi YQ, Lokuta AJ, Greaser ML & Walker JW (1997). Arachidonic acid stimulates protein kinase C-epsilon redistribution in heart cells. *J Cell Sci* **110,** 1625-1634.

Iemitsu M, Shimojo N, Maeda S, Irukayama-Tomobe Y, Sakai S, Ohkubo T, Tanaka Y & Miyauchi T (2008). The benefit of medium-chain triglyceride therapy on the cardiac function of SHRs is associated with a reversal of metabolic and signaling alterations. *Am J Physiol-Heart C* **295,** H136-H144.

Inagaki K, Chen L, Ikeno F, Lee FH, Imahashi K, Bouley DM, Rezaee M, Yock PG, Murphy E & Mochly-Rosen D (2003). Inhibition of delta-protein kinase C protects against reperfusion injury of the ischemic heart in vivo. *Circulation* **108,** 2304-2307.

Inagaki K, Koyanagi T, Berry NC, Sun L & Mochly-Rosen D (2008). Pharmacological inhibition of epsilon-protein kinase C attenuates cardiac fibrosis and dysfunction in hypertension-induced heart failure. *Hypertension* **51,** 1565-1569.

Irie H, Krukenkamp IB, Brinkmann JF, Gaudette GR, Saltman AE, Jou W, Glatz JF, Abumrad NA & Ibrahimi A (2003). Myocardial recovery from ischemia is impaired in CD36-null mice and restored by myocyte CD36 expression or medium-chain fatty acids. *Proc Natl Acad Sci U S A* **100,** 6819-6824.

Jacob AI, Horovitz-Fried M, Aga-Mizrachi S, Brutman-Barazani T, Okhrimenko H, Zick Y, Brodie C & Sampson SR (2010). The regulatory domain of protein kinase C delta positively regulates insulin receptor signaling. *J Mol Endocrinol* **44,** 155-169.

Karpe F, Dickmann JR & Frayn KN (2011). Fatty acids, obesity, and insulin resistance: time for a reevaluation. *Diabetes* **60,** 2441-2449.

Klevstig MJ, Markova I, Burianova J, Kazdova L, Pravenec M, Novakova O & Novak F (2011). Role of FAT/CD36 in novel PKC isoform activation in heart of spontaneously hypertensive rats. *Mol Cell Biochem* **357,** 163-169.

Kolar F, Jezkova J, Balkova P, Breh J, Neckar J, Novak F, Novakova O, Tomasova H, Srbova M, Ost'adal B, Wilhelm J & Herget J (2007). Role of oxidative stress in PKC-delta upregulation and cardioprotection induced by chronic intermittent hypoxia. *Am J Physiol-Heart C* **292,** H224-H230.

Kolar F & Parratt JR (1997). Antiarrhythmic effect of ischemic preconditioning in hearts of spontaneously hypertensive rats. *Exp Clin Cardiol* **2,** 124-127.

Koonen DPY, Febbraio M, Bonnet S, Nagendran J, Young ME, Michelakis ED & Dyck JRB (2007). CD36 expression contributes to age-induced cardiomyopathy in mice. *Circulation* **116,** 2139-2147.

Koya D & King GL (1998). Protein kinase C activation and the development of diabetic complications. *Diabetes* **47,** 859-866.

Kuang M, Febbraio M, Wagg C, Lopaschuk GD & Dyck JRB (2004). Fatty acid translocase/CD36 deficiency does not energetically or functionally compromise hearts before or after ischemia. *Circulation* **109,** 1550-1557.

Labarthe F, Khairallah M, Bouchard B, Stanley WC & Des Rosiers C (2005). Fatty acid oxidation and its impact on response of spontaneously hypertensive rat hearts to an adrenergic stress: benefits of a medium-chain fatty acid. *Am J Physiol-Heart C* **288,** H1425-H1436.

Lam A & Lopaschuk GD (2007). Anti-anginal effects of partial fatty acid oxidation inhibitors. *Curr Opin Pharmacol***7,** 179-185.

Lange SA, Wolf B, Schober K, Wunderlich C, Marquetant R, Weinbrenner C & Strasser RH (2007). Chronic angiotensin II receptor blockade induces cardioprotection during ischemia by increased PKC-epsilon expression in the mouse heart. *J Cardiovasc Pharm* **49,** 46-55.

Laybutt DR, Schmitz-Peiffer C, Saha AK, Ruderman NB, Biden TJ & Kraegen EW (1999). Muscle lipid accumulation and protein kinase C activation in the insulin-resistant chronically glucose-infused rat. *Am J Physiol-Endoc M* **277,** E1070-E1076.

Leenen FHH & Yuan BX (2001). Mortality after coronary artery occlusion in different models of cardiac hypertrophy in rats. *Hypertension* **37,** 209-215.

Liggett SB, Tepe NM, Lorenz JN, Canning AM, Jantz TD, Mitarai S, Yatani A & Dorn GW (2000). Early and delayed consequences of beta(2)-adrenergic receptor overexpression in mouse hearts - Critical role for expression level. *Circulation* **101,** 1707-1714.

Lissandron V & Zaccolo M (2006). Compartmentalized cAMP/PKA signalling regulates cardiac excitation-contraction coupling. *J Muscle Res Cell M* **27,** 399-403.

Lu YJ, Zhang Y, Shan HL, Pan ZW, Li XL, Li BX, Xu CQ, Zhang BS, Zhang FM, Dong DL, Song WQ, Qiao GF & Yang BF (2009). MicroRNA-1 downregulation by propranolol in a rat model of myocardial infarction: a new mechanism for ischaemic cardioprotection. *Cardiovasc Res* **84,** 434-441.

Luiken JJFP, Arumugam Y, Dyck DJ, Bell RC, Pelsers MML, Turcotte LP, Tandon NN, Glatz JFC & Bonen A (2001). Increased rates of fatty acid uptake and plasmalemmal fatty acid transporters in obese Zucker rats. *J Biol Chem* **276,** 40567-40573.

Luiken JJFP, Coort SLM, Koonen DPY, van der Horst DJ, Bonen A, Zorzano A & Glatz JFC (2004). Regulation of cardiac long-chain fatty acid and glucose uptake by translocation of substrate transporters. *Pflug Arch Eur J Phy* **448,** 1-15.

Luiken JJFP, Omens DM, Habets DDJ, van der Zon GCM, Coumans WA, Schwenk RW, Bonen A & Glatz JFC (2009). Permissive action of protein kinase C-zeta in insulin-induced CD36-and GLUT4 translocation in cardiac myocytes. *J Endocrinol* **201,** 199-209.

Maier T, Schreckenberg R & Schluter KD (2009). Effect of preischemic beta-adrenoceptor stimulation on postischemic contractile dysfunction. *Life Sci* **84,** 437-443.

Malhotra A, Begley R, Kang BPS, Rana I, Liu J, Yang GP, Mochly-Rosen D & Meggs LG (2005). PKC-epsilon-dependent survival signals in diabetic hearts. *Am J Physiol-Heart C* **289,** H1343-H1350.

Martin C, Chevrot M, Poirier H, Passilly-Degrace P, Niot I & Besnard P (2011). CD36 as a lipid sensor. *Physiol Behav* **105,** 36-42.

Martins AR, Nachbar RT, Gorjao R, Vinolo MA, Festuccia WT, Lambertucci RH, Cury-Boaventura MF, Silveira LR, Curi R & Hirabara SM (2012). Mechanisms underlying skeletal muscle insulin resistance induced by fatty acids: importance of the mitochondrial function. *Lipids Health Dis* **11,** 30.

Matsumori Y, Ohyanagi M, Kawamoto H, Shibata R & Iwasaki T (1989). Intracellular-distribution of cardiac beta-adrenoceptors in Shr and Wky. *Jpn Circ J* **53,** 113-120.

Mayr M, Chung YL, Mayr U, McGregor E, Troy H, Baier G, Leitges M, Dunn MJ, Griffiths JR & Xu QB (2004). Loss of PKC-delta alters cardiac metabolism. *Am J Physiol-Heart C* **287,** H937-H945.

Mellor KM, Ritchie RH & Delbridge LMD (2010). Reactive oxygen species and insulin-resistant cardiomyopathy. *Clin Exp Pharmacol P* **37,** 222-228.

Mulvany MJ & Nyborg N (1980). An increased calcium sensitivity of mesenteric resistance vessels in young and adult spontaneously hypertensive rats. *Br J Pharmac* **71,** 585-596.

Murphy E & Steenbergen C (2008). Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol Rev* **88,** 581-609.

Neckar J, Silhavy J, Zidek V, Landa V, Mlejnek P, Simakova M, Seidman JG, Seidman C, Kazdova L, Klevstig M, Novak F, Vecka M, Papousek F, Houstek J, Drahota Z, Kurtz TW, Kolar F & Pravenec M (2012). CD36 overexpression predisposes to arrhythmias but reduces infarct size in spontaneously hypertensive rats: gene expression profile analysis. *Physiol Genomics* **44,** 173-182.

Newton AC (2010). Protein kinase C: poised to signal. *Am J Physiol-Endoc M* **298,** E395-E402.

Niu WY, Bilan PJ, Yu JN, Gao J, Boguslavsky S, Schertzer JD, Chu GL, Yao Z & Klip A (2011). PKC epsilon regulates contraction-stimulated GLUT4 traffic in skeletal muscle cells. *J Cell Physiol* **226,** 173-180.

Ogita Z & Markert CL (1979). Miniaturized system for electrophoresis on polyacrylamide gels. *Anal Biochem* **99,** 233-241.

Okamoto K & Aoki K (1963). Development of a strain of spontaneously hypertensive rats. *Jpn Circ J* **27,** 282-293.

Okumura S, Vatner DE, Kurotani R, Bai Y, Gao SM, Yuan ZR, Iwatsubo K, Ulucan C, Kawabe J, Ghosh K, Vatner SF & Ishikawa Y (2007). Disruption of type 5 adenylyl cyclase enhances desensitization of cyclic adenosine monophosphate signal and increases Akt signal with chronic catecholamine stress. *Circulation* **116,** 1776-1783.

Oliver MF (2001). Prevention of ventricular fibrillation during acute myocardial ischemia: control of free fatty acids. *J Cardiovasc Pharmacol Ther* **6,** 213-217.

Oliver MF (2006). Sudden cardiac death: the lost fatty acid hypothesis. *Qjm-Int J Med* **99,** 701-709.

Ouwens DM, Diamant M, Fodor M, Habets DDJ, Pelsers MMAL, El Hasnaoui M, Dang ZC, van den Brom CE, Vlasblom R, Rietdijk A, Boer C, Coort SLM, Glatz JFC & Luiken JJFP (2007). Cardiac contractile dysfunction in insulin-resistant rats fed a

high-fat diet is associated with elevated CD36-mediated fatty acid uptake and esterification. *Diabetologia* **50,** 1938-1948.

Oxman T, Arad M, Klein R, Avazov N & Rabinowitz B (1997). Limb ischemia preconditions the heart against reperfusion tachyarrhythmia. *Am J Physiol-Heart C* **273,** H1707-H1712.

Penson P, Ford W, Kidd E & Broadley K (2008). Activation of beta-adrenoceptors mimics preconditioning of rat-isolated atria and ventricles against ischaemic contractile dysfunction. *N-S Arch Pharmacol* **378,** 589-597.

Pepe S & McLennan PL (2002). Cardiac membrane fatty acid composition modulates myocardial oxygen consumption and postischemic recovery of contractile function. *Circulation* **105,** 2303-2308.

Peterson GL (1977). Simplification of protein assay method of Lowry et al - which is more generally applicable. *Anal Biochem* **83,** 346-356.

Pfeffer MA, Pfeffer JM, Weiss AK & Frohlich ED (1977). Development of SHR hypertension and cardiac hypertrophy during prolonged beta blockade. *Am J Physiol* **232,** H639-H644.

Pietka TA, Sulkin MS, Kuda O, Wang W, Zhou D, Yamada KA, Yang K, Su X, Gross RW, Nerbonne JM, Efimov IR & Abumrad NA (2012). CD36 influences myocardial Ca2+ homeostasis and phospholipid metabolism: Conduction anomalies in CD36 deficient mice during fasting. *J Biol Chem* **287,** 38901-38912.

Pravenec M, Gauguier D, Schott JJ, Buard J, Kren V, Bila V, Szpirer C, Szpirer J, Wang JM, Huang HM, Stlezin E, Spence MA, Flodman P, Printz M, Lathrop GM, Vergnaud G & Kurtz TW (1995). Mapping of quantitative trait loci for blood-pressure and cardiac mass in the rat by genome scanning of recombinant inbred strains. *J Clin Invest* **96,** 1973-1978.

Pravenec M, Churchill PC, Churchill MC, Viklicky O, Kazdova L, Aitman TJ, Petretto E, Hubner N, Wallace CA, Zimdahl H, Zidek V, Landa V, Dunbar J, Bidani A, Griffin K, Qi N, Maxova M, Kren V, Mlejnek P, Wang J & Kurtz TW (2008a). Identification of renal Cd36 as a determinant of blood pressure and risk for hypertension. *Nat Genet* **40,** 952-954.

Pravenec M, Kajiya T, Zidek V, Landa V, Mlejnek P, Simakova M, Silhavy J, Malinska H, Oliyarnyk O, Kazdova L, Fan JL, Wang JM & Kurtz TW (2011). Effects of human C-reactive protein on pathogenesis of features of the metabolic syndrome. *Hypertension* **57,** 731-737.

Pravenec M, Kazdova L, Landa V, Zidek V, Mlejnek P, Simakova M, Jansa P, Forejt J, Kren V, Krenova D, Qi N, Wang JM, Chan D, Aitman TJ & Kurtz TW (2008b). Identification of mutated Srebf1 as a QTL influencing risk for hepatic steatosis in the spontaneously hypertensive rat. *Hypertension* **51,** 148-153.

Pravenec M & Kurtz TW (2010). Recent advances in genetics of the spontaneously hypertensive rat. *Curr Hypertens Rep* **12,** 5-9.

Pravenec M, Landa V, Zidek V, Musilova A, Kren V, Kazdova L, Aitman TJ, Glazier AM, Ibrahimi A, Abumrad NA, Qi NN, Wang JM, St Lezin EM & Kurtz TW (2001).

Transgenic rescue of defective Cd36 ameliorates insulin resistance in spontaneously hypertensive rats. *Nat Genet* **27,** 156-158.

Pravenec M, Zidek V, Simakova M, Kren V, Krenova D, Horky K, Jachymova M, Mikova B, Kazdova L, Aitman TJ, Churchill PC, Webb RC, Hingarh NH, Yang Y, Wang JM, St Lezin EM & Kurtz TW (1999). Genetics of Cd36 and the clustering of multiple cardiovascular risk factors in spontaneous hypertension. *J Clin Invest* **103,** 1651-1657.

Purushothaman S, Nair RR, Harikrishnan VS & Fernandez AC (2011). Temporal relation of cardiac hypertrophy, oxidative stress, and fatty acid metabolism in spontaneously hypertensive rat. *Mol Cell Biochem* **351,** 59-64.

Rafiee P, Shi Y, Kong XR, Pritchard KA, Tweddell JS, Litwin SB, Mussatto K, Jaquiss RD, Su JD & Baker JE (2002). Activation of protein kinases in chronically hypoxic infant human-and rabbit hearts - Role in cardioprotection. *Circulation* **106,** 239-245.

Ragheb R, Shanab GML, Medhat AM, Seoudi DM, Adeli K & Fantus IG (2009). Free fatty acid-induced muscle insulin resistance and glucose uptake dysfunction: Evidence for PKC activation and oxidative stress-activated signaling pathways. *Biochem Bioph Res Co* **389,** 211-216.

Rauch B & Senges J (2012). The effects of supplementation with omega-3 polyunsaturated Fatty acids on cardiac rhythm: anti-arrhythmic, pro-arrhythmic, both or neither? It depends. *Front Physiol* **3,** 57.

Ravingerova T, Bernatova I, Matejikova J, Ledvenyiova V, Nemcekova M, Pechanova O, Tribulova N & Slezak J (2011). Impaired cardiac ischemic tolerance in spontaneously hypertensive rats is attenuated by adaptation to chronic and acute stress. *Exp Clin Cardiol* **16,** E23-E29.

Reaven GM (1993). Role of insulin resistance in human-disease (syndrome-X) - an expanded definition. *Annu Rev Med* **44,** 121-131.

Reaven GM & Chang H (1991). Relationship between blood-pressure, plasma-insulin and triglyceride concentration, and insulin action in spontaneous hypertensive and Wistar-Kyoto rats. *Am J Hypertens* **4,** 34-38.

Rizzoni D, Pasini E, Flati V, Rodella LF, Paiardi S, Assanelli D, De Ciuceis C, Porteri E, Boari GEM, Rezzani R, Speca S, Favero G, Martinotti S, Toniato E, Platto C & Agabiti-Rosei E (2008). Angiotensin receptor blockers improve insulin signaling and prevent microvascular rarefaction in the skeletal muscle of spontaneously hypertensive rats. *J Hypertens* **26,** 1595-1601.

Rockman HA, Koch WJ & Lefkowitz RJ (2002). Seven-transmembrane-spanning receptors and heart function. *Nature* **415,** 206-212.

Roth DM, Bayat H, Drumm JD, Gao MH, Swaney JS, Ander A & Hammond HK (2002). Adenylyl cyclase increases survival in cardiomyopathy. *Circulation* **105,** 1989-1994.

Rybin VO & Steinberg SF (1994). Protein-kinase-C isoform expression and regulation in the developing rat-heart. *Circ Res* **74,** 299-309.

Samovski D, Su X, Xu YC, Abumrad NA & Stahl PD (2012). Insulin and AMPK regulate FA translocase/CD36 plasma membrane recruitment in cardiomyocytes via Rab GAP AS160 and Rab8a Rab GTPase. *J Lipid Res* **53,** 709-717.

Sampson SR & Cooper DR (2006). Specific protein kinase C isoforms as transducers and modulators of insulin signaling. *Mol Genet Metab* **89,** 32-47.

Sen S, Tarazi RC, Khairallah PA, Bumpus FM (1974). Cardiac hypertrophy in spontaneously hypertensive rats. *Circ Res* **35,** 775-781.

Shimojo N, Miyauchi T, Iemitsu M, Irukayama-tomobe Y, Maeda S, Ohkubo T, Tanaka Y, Goto K & Yamaguchi I (2004). Effects of medium-chain triglyceride (MCT) application to SHR on cardiac function, hypertrophy and expression of endothelin-1 mRNA and other genes. *J Cardiovasc Pharm* **44,** S181-S185.

Schmitz-Peiffer C, Laybutt DR, Burchfield JG, Gurisik E, Narasimhan S, Mitchell CJ, Pedersen DJ, Braun U, Cooney GJ, Leitges M & Biden TJ (2007). Inhibition of PKC epsilon improves glucose-stimulated insulin secretion and reduces insulin clearance. *Cell Metab* **6,** 320-328.

Schwartz GJ, Fu J, Astarita G, Li XS, Gaetani S, Campolongo P, Cuomo V & Piomelli D (2008). The lipid messenger OEA links dietary fat intake to satiety. *Cell Metab* **8,** 281-288.

Schwenk RW, Luiken JJFP, Bonen A & Glatz JFC (2008). Regulation of sarcolemmal glucose and fatty acid transporters in cardiac disease. *Cardiovasc Res* **79,** 249-258.

Silverstein RL & Febbraio M (2009). CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. *Sci Signal* **2,** doi: 10.1126/scisignal.272re3.

Silverstein RL, Li W, Park YM & Rahaman SO (2010). Mechanisms of cell signaling by the scavenger receptor CD36: implications in atherosclerosis and thrombosis. *Trans Am Clin Climatol Assoc* **121,** 206-220.

Skalhegg BS & Tasken K (2000). Specificity in the cAMP/PKA signaling pathway. Differential expression, regulation, and subcellular localization of subunits of PKA. *Front Biosci* **5,** D678-D693.

Slee EL, McLennan PL, Owen AJ & Theiss ML (2010). Low dietary fish-oil threshold for myocardial membrane n-3 PUFA enrichment independent of n-6 PUFA intake in rats. *J Lipid Res* **51,** 1841-1848.

Slotkin TA, Auman JT & Seidler FJ (2003). Ontogenesis of beta-adrenoceptor signaling: Implications for perinatal physiology and for fetal effects of tocolytic drugs. *J Pharmacol Exp Ther* **306,** 1-7.

Smith BK, Jain SS, Rimbaud S, Dam A, Quadrilatero J, Ventura-Clapier R, Bonen A & Holloway GP (2011). FAT/CD36 is located on the outer mitochondrial membrane, upstream of long-chain acyl-CoA synthetase, and regulates palmitate oxidation. *Biochem J* **437,** 125-134.

Smith J, Su X, El-Maghrabi R, Stahl PD & Abumrad NA (2008). Opposite regulation of CD36 ubiquitination by fatty acids and insulin - Effects on fatty acid uptake. *J Biol Chem* **283,** 13578-13585.

Soto D, De Arcangelis V, Zhang J & Xiang Y (2009). Dynamic protein kinase A activities induced by beta-adrenoceptors dictate signaling propagation for substrate phosphorylation and myocyte contraction. *Circ Res* **104,** 770-779.

Stahrenberg R, Edelmann F, Mende M, Kockskamper A, Dungen HD, Scherer M, Kochen MM, Binder L, Herrmann-Lingen C, Gelbrich G, Hasenfuss G, Pieske B & Wachter R (2010). Association of glucose metabolism with diastolic function along the diabetic continuum. *Diabetologia* **53,** 1331-1340.

Stanley WC, Recchia FA & Lopaschuk GD (2005). Myocardial substrate metabolism in the normal and failing heart. *Physiol Rev* **85,** 1093-1129.

Steinbusch LKM, Schwenk RW, Ouwens DM, Diamant M, Glatz JFC & Luiken JJFP (2011). Subcellular trafficking of the substrate transporters GLUT4 and CD36 in cardiomyocytes. *Cell Mol Life Sci* **68,** 2525-2538.

Sung MMY, Koonen DPY, Soltys CLM, Jacobs RL, Febbraio M & Dyck JRB (2011). Increased CD36 expression in middle-aged mice contributes to obesity-related cardiac hypertrophy in the absence of cardiac dysfunction. *J Mol Med* **89,** 459-469.

Takahashi T, Tang T, Lai NC, Roth DM, Rebolledo B, Saito M, Lew WYW, Clopton P & Hammond HK (2006). Increased cardiac adenylyl cyclase expression is associated with increased survival after myocardial infarction. *Circulation* **114,** 388-396.

Tang T, Gao MH, Lai NC, Firth AL, Takahashi T, Guo T, Yuan JXJ, Roth DM & Hammond HK (2008). Adenylyl cyclase type 6 deletion decreases left ventricular function via impaired calcium handling. *Circulation* **117,** 61-69.

Tang YQ, Wang MH, Le XY, Meng JN, Huang L, Yu P, Chen J & Wu P (2011). Antioxidant and cardioprotective effects of Danshensu (3-(3, 4-dihydroxyphenyl)-2-hydroxy-propanoic acid from Salvia miltiorrhiza) on isoproterenol-induced myocardial hypertrophy in rats. *Phytomedicine* **18,** 1024-1030.

Tasken K & Aandahl EM (2004). Localized effects of cAMP mediated by distinct routes of protein kinase A. *Physiol Rev* **84,** 137-167.

Tong H, Bernstein D, Murphy E & Steenbergen C (2005). The role of beta-adrenergic receptor signaling in cardioprotection. *FASEB J* **19,** 983-985.

Turban S & Hajduch E (2011). Protein kinase C isoforms: mediators of reactive lipid metabolites in the development of insulin resistance. *Febs Lett* **585,** 269-274.

Turer AT, Stevens RD, Bain JR, Muehlbauer MJ, van der Westhuizen J, Mathew JP, Schwinn DA, Glower DD, Newgard CB & Podgoreanu MV (2009). Metabolomic profiling reveals distinct patterns of myocardial substrate use in humans with coronary artery disease or left ventricular dysfunction during surgical ischemia/reperfusion. *Circulation* **119,** 1736-U1788.

Tvrzicka E, Vecka M, Stankova B & Zak A (2002). Analysis of fatty acids in plasma lipoproteins by gas chromatography-flame ionization detection Quantitative aspects. *Anal Chim Acta* **465,** 337-350.

Vandervusse GJ, Glatz JFC, Stam HCG & Reneman RS (1992). Fatty-acid homeostasis in the normoxic and ischemic heart. *Physiol Rev* **72,** 881-940.

Wang PP, Lloyd SG, Zeng HD, Bonen A & Chatham JC (2005). Impact of altered substrate utilization on cardiac function in isolated hearts from Zucker diabetic fatty rats. *Am J Physiol-Heart C* **288,** H2102-H2110.

Waraich RS, Weigert C, Kalbacher H, Hennige AM, Lutz SZ, Haring HU, Schleicher ED, Voelter W & Lehmann R (2008). Phosphorylation of Ser(357) of rat insulin receptor substrate-1 mediates adverse effects of protein kinase C-delta on insulin action in skeletal muscle cells. *J Biol Chem* **283,** 11226-11233.

Woods A, Dickerson K, Heath R, Hong SP, Momcilovic M, Johnstone SR, Carlson M & Carling D (2005). Ca2+/calmodulin-dependent protein kinase kinase-beta acts upstream of AMP-activated protein kinase in mammalian cells. *Cell Metab* **2,** 21-33.

Workman AJ (2010). Cardiac adrenergic control and atrial fibrillation. *N-S Arch Pharmacol* **381,** 235-249.

Wu GY, Toyokawa T, Hahn H & Dorn GW (2000). epsilon Protein kinase C in pathological myocardial hypertrophy - Analysis by combined transgenic expression of translocation modifiers and G alpha(q). *J Biol Chem* **275,** 29927-29930.

Xiang Y & Kobilka BK (2003). Myocyte adrenoceptor signaling pathways. *Science* **300,** 1530-1532.

Xiao RP, Zhu WZ, Zheng M, Cao CM, Zhang YY, Lakatta EG & Han Q (2006). Subtype-specific alpha(1)- and beta-adrenoceptor signaling in the heart. *Trends Pharmacol Sci* **27,** 330-337.

Ytrehus K, Liu YG & Downey JM (1994). Preconditioning protects ischemic rabbit heart by protein-kinase-C activation. *Am J Physiol* **266,** H1145-H1152.

Zeghichi-Hamri S, de Lorgeril M, Salen P, Chibane M, de Leiris J, Boucher F & Laporte F (2010). Protective effect of dietary n-3 polyunsaturated fatty acids on myocardial resistance to ischemia-reperfusion injury in rats. *Nutr Res* **30,** 849-857.

Zhang L, Xu CQ, Hong YA, Zhang JL, Liu Y, Zhao M, Cao YX, Lu YJ, Yang BF & Shan HL (2010). Propranolol regulates cardiac transient outward potassium channel in rat myocardium via cAMP/PKA after short-term but not after long-term ischemia. *N-S Arch Pharmacol* **382,** 63-71.

Zhu WZ, Zheng M, Koch WJ, Lefkowitz RJ, Kobilka BK & Xiao RP (2001). Dual modulation of cell survival and cell death by beta(2)-adrenergic signaling in adult mouse cardiac myocytes. *P Natl Acad Sci USA* **98,** 1607-1612.

Zimmet P, Magliano D, Matsuzawa Y, Alberti G & Shaw J (2005). The metabolic syndrome: a global public health problem and a new definition. *J Atheroscler Thromb* **12,** 295-300.

Zolk O, Kouchi I, Schnabel P & Bohm M (2000). Heterotrimeric G proteins in heart disease. *Can J Physiol Pharm* **78,** 187-198.

8 SUPPLEMENTS

Supplement I

Klevstig MJ, Markova I, Burianova J, Kazdova L, Pravenec M, Novakova O, Novak F (2011). Role of FAT/CD36 in novel PKC isoform activation in heart of spontaneously hypertensive rats.*Mol Cell Biochem* **357,** 163-169. IF=2.057

Supplement II

Neckar J, Silhavy J, Zidek V, Landa V, Mlejnek P, Simakova M, Seidman JG, Seidman C, Kazdova L, **Klevstig M**, Novak F, Vecka M, Papousek F, Houstek J, Drahota Z, Kurtz TW, Kolar F & Pravenec M (2012). CD36 overexpression predisposes to arrhythmias but reduces infarct size in spontaneously hypertensive rats: gene expression profile analysis. *Physiol Genomics* **44,** 173-182. IF=2.735

Supplement III

Klevstig M, Manakov D, Kasparova D, Brabcova I, Papousek F, Zurmanova J, Zidek V, Silhavy J, Neckar J, Pravenec M, Kolar F, Novakova O, Novotny J (2013). Transgenic rescue of defective Cd36 enhances myocardial adenylyl cyclase signaling in spontaneously hypertensive rats. *Pflug Arch Eur J Phy* (Accepted for publication). IF=4.463