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Non-canonical bioenergetics of the cell

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CONTENT

SOUHRN

Rakovinné buňky jsou charakteristické netypickými bioenergetickými vlastnostmi, jako je zvýšený metabolizmus glukózy a neefektivní oxidativní fosforylace. Přesný mechanizmus této metabolické proměny není doposud znám. Cílem tohoto projektu bylo ujasnit, jak prostředí tumoru ovlivňuje fungování mitochondriální oxidativní fosforylace. Zaměřovali jsme se na dvě základní podmínky, tj. deprivace glukózy a kyslíku, a analyzovali růst buněk, bioenergetické vlastnosti a expresi proteinů oxidativní fosforylace. Jako experimentální model jsme použili rakovinné buňky tumoru mléčné žlázy (HTB-126), které jsme porovnávali s příslušnou kontrolní linií (HTB-125), protože prostředí karcinomu prsu bývá často hypoxické a aglykemické. Výsledky této práce jasně ukazují závislost mitochondriální bioenergetiky na přítomnosti glukózy u rakovinných buněk. Mitochondriální respirace je regulována glukózou na několika úrovních, jako je Crabtree efekt, odvádění energetického substrátu z mitochondrií k podpoře buněčného růstu a proliferace a na úrovni biogeneze mitochondrií. Fungování oxidativní fosforylace je jasně ovlivněna přítomností glukózy i kyslíku. Součástí přizpůsobení se hypoxii je potlačení oxidativní fosforylace přítomností glukózy mnohem více, než v normoxii. Naše data ukazují, že energetické vlastnosti rakovinných buněk jsou regulovány vlivem buněčného okolí, konkrétně relativním vlivem přítomnosti glukózy a kyslíku.

Mitochondriální odpřahující proteiny jsou protein vnitřní mitochondriální membrány schopné svojí aktivitou odpřáhnout mitochondrie, tj. odpřáhnout respiraci od ATP syntézy. Původně proklamovaná tkáňová distribuce je nyní považována za zastaralou. Například, UCP1, původní „thermogenin“ hnědé tukové tkáně byl nalezen i v dalších tkáních, podobně jako mozkově-specifické izoformy UCP4 a UCP5. Ve druhé části práce jsou diskutovány výsledky expresní profily UCPn izoforem ve vybraných tkáních myši a potkana.

Mitochondrie se rovněž účastní regulace apoptózy, která je přirozený, geneticky kontrolovaný proces buněčné smrti. Přibývá poznatků o apoptotických drahách a zúčastněných proteinech; například dráhy aktivující kaspázy společně s proteiny rodiny Bcl jsou poměrně dobře charakterizované. Naproti tomu, existují apoptotické dráhy méně prozkoumané, konkrétně zahrnující homologní proteiny apoptotické nukleázy DFF, CIDE, nezávislé na kaspázách, které popisuje třetí část této práce.

SUMMARY

Cancer cells generally present abnormal bioenergetic properties including an elevated glucose uptake, a high glycolysis and a poorly efficient oxidative phosphorylation system. However, the determinants of cancer cells metabolic reprogramming remain unknown. The main question in this project was how environmental conditions *in vivo* can influence functioning of mitochondrial OXPHOS, because details of mitochondrial bioenergetics of cancer cells is poorly documented. We have combined two conditions, namely glucose and oxygen deprivation, to measure their potential interaction. We examined the impact of glucose deprivation and oxygen deprivation on cell survival, overall bioenergetics and OXPHOS protein expression. As a model, we have chosen a human breast carcinoma (HTB-126) and appropriate control (HTB-125) cultured cells, as large fraction of breast malignancies exhibit hypoxic tumor regions with low oxygen concentrations and poor glucose delivery. The results demonstrate that glucose presence or absence largely influence functioning of mitochochondrial oxidative phosphorylation. The level of mitochondrial respiration capacity is regulated by glucose; by Crabtree effect, by energy substrate channeling towards anabolic pathways that support cell growth and by mitochondrial biogenesis pathways. Both oxygen deprivation and glucose deprivation can remodel the OXPHOS system, albeit in opposite directions. As an adaptative response to hypoxia, glucose inhibits mitochondrial oxidative phosphorylation to the larger extent than in normoxia. We concluded that the energy profile of cancer cells can be determined by specific balance between two main environmental stresses, glucose and oxygen deprivation. Thus, variability of intratumoral environment might explain the variability of cancer cells´ bioenergetic profile.

Mitochondrial uncoupling proteins are proteins of inner mitochondrial membrane that uncouple respiration from ATP synthesis by their protonophoric activity. Originally determined tissue distribution seems to be invalid, since novel findings show that UCP1 is not restricted exclusively to brown fat and that originally considered brain-specific isoforms UCP4 and UCP5 might have wider tissue distribution. Hence, in second part of thesis, I discuss consequences of findings of UCPn transcripts in the studied mouse and rat tissues.

Apoptosis is a natural, genetically controlled process of cell elimination. The mechanisms of its activation and regulation is a fundamental scientific question and growing body of evidence reveal further molecular pathways of apoptotic machinery. The well-known caspase activation cascade along with pro- and anti-apoptotic members of BCL family is the basic structure of apoptotic machinery. However, side pathways of executive steps of apoptosis have been revealed, namely proteins with homology to nuclease DFF responsible for apoptotic DNA cleavage. In this part of thesis, we tried to elucidate another apoptotic pathway connected to mitochondria, independent of caspases.

1. INTRODUCTION

1.1. Significance of non-canonical bioenergetics

Canonical bioenergetics has explained on the basis of Mitchell chemiosmotic theory the mechanisms of ATP synthesis in mitochondria, chloroplasts, bacteria, and other entities, including the essential coupling of the electron flow through the complexes of the respiratory chain and concomitant proton pumping with the ATP synthesis by the ATP synthase. Indeed protons are the intermediates of this coupling and a physical quantity called gradient of electrochemical potential of protons or the protonmotive force, □p (when divided by Faraday constant and expressed in mV, according to Nobel Prize laureate Peter Mitchell). Yet, the canonical bioenergetics has recognized detail structure of huge respiratory chain complexes (pioneered by Hartmudt Michel as Nobel Prize laureate), rotatory mechanism of ATP synthesis as well as structure of the ATP synthase (other 1997 Nobel laureates, John Walker, Paul D Boyer) and numerous other findings including biogenesis and assembly of these complexes.

Nevertheless, canonical bioenergetics turned out to be unable to explain various physiological and pathological phenomena. Non-canonical bioenergetics concerns with those physiological and pathophysiological situations under which ATP synthesis is suppressed. The early findings of Otto Warburg (actually the first Nobel Prize laureate in bioenergetics) led to his hypothesis on the exclusively glycolytic phenotype of tumor cells. Since 1961 a brown adipose tissue has been discovered, a tissue deliberately dissipating energy into the heat. Subsequent discoveries of mitochondrial uncoupling protein-1, originally found specific for brown adipose tissue (today known with extended expression pattern), and the whole subfamily of uncoupling proteins (UCP2 to UCP5 plus three plant isoforms) have opened a new chapter of non-canonical bioenergetics, recognizing that a mild uncoupling may physiologically regulate superoxide formation of mitochondria, hence the redox signaling in mitochondria and cell (see below). A self-standing field concerns with the "tax for life", i.e. inevitable inherent formation of superoxide within the respiratory chain Complexes I and III. Superoxide is a species at the top of cascade of reactive oxygen species (ROS), creating oxidative stress if elevated. A constant ROS formation as an inevitable byproduct of respiration leads to aging and if pathogenically accelerated it causes numerous diseases. This field of oxidative stress however, has expanded into a vast field of redox regulations, recognizing "physiologically slight" ROS elevations as fundamentally initiating certain information signaling pathways. An exemplar prototype concerns with ROS-initiation of hypoxia-mediated-factor-signaling, in fact representing oxygen

sensing in hypoxia by the respiratory chain. Thus non-canonical bioenergetics of the cell is the science for years to come.

1.2. Energy metabolism of cancer cells

Based on proposal of Warburg (Warburg 1956), glycolysis of cancer cells is often highly enhanced even in the presence of oxygen, resulting in an excessive lactate production (Warburg effect, aerobic glycolysis). These findings lead investigators to consider oxidative phosphorylation of cancer cells to be necessarily impaired during carcinogenesis. Aerobic respiration from glucose produces 38 mol of ATP, 19 times more than glycolysis, although the rate of ATP production by the OXPHOS is less rapid than glycolysis *(Pfeiffer et al. 2001)*. However, due to the high glycolytic turnover that cancer cells maintain, ATP production is sufficient to support the cell growth. Human living tumoral tissues exhibit elevated glucose uptake when compared to surrounding normal tissue *(Nakata et al. 2001; Mankoff et al. 2007)*. It must be stressed that cellular metabolism, and particularly oxidative metabolism, are totally subverted to the needs of cancer cells in which proliferation is the primary function. Despite obvious disadvantage in term of producing ATP, elevated glucose metabolism is considered to be somehow supportive in cell growth. Enhanced glycolysis is also considered a preconditioning for hypoxia. Indeed, in many aspects, Warburg effect resembles metabolic switch occurring as an adaptation to anaerobic conditions. Some tumor cells are reported to be strictly dependent on glycolytical ATP *(Fantin et al. 2006)*, so that proliferation with OXPHOS-derived ATP exclusively is attenuated.

1.3. Variability of metabolic phenotypes in cancer cells

Despite the existence of aerobic glycolysis in numerous tumor types, OXPHOS activity within tumor cells is not necessarily attenuated considering numerous reports indicating strong OXPHOS activity and dependency on aerobic ATP production. Therefore, Warburg hypothesis cannot be interpreted so strictly anymore, despite the indisputable fraction of tumors with aerobic glycolysis; mitochondria of cancer cells are not dysfunctional in general, rather it operates at a low-capacity there. There are numerous reports demonstrating that oxidative phosphorylation prevails over glycolysis *(Guppy et al. 2002; Nakashima et al. 1984; Rodríguez-Enríquez et al. 2006)*. Further studies evidenced a class of tumor cell lines in which the oxidative metabolism prevails over glycolysis. This situation has been extensively reviewed *(Moreno-Sánchez et al. 2007)*, and the authors even conclude that "high glycolysis" is not a prerequisite of all cancer cells but could be acquired during the highest proliferative activity and/or in response to stringent micro-environmental conditions, such as intermittent hypoxia. Accordingly, a critical review of

numerous studies comparing cancer cells with normal tissues concluded that several tumors derive most of their ATP from mitochondrial oxidative phosphorylation, in striking contrast to Warburg's hypothesis *(Zu and Guppy 2004)*. Therefore, due to the genetic heterogeneity of tumor cells, OXPHOS capacity should be experimentally evaluated for each particular tumor type, to assess whether the enhanced glycolysis is indeed accompanied by a significant depression of mitochondrial function.

1.4. Crabtree effect

It was evidenced, that tumor cells are able to react rapidly to a presence of exogenous fuel. Acute inhibition of respiration by exogenous hexoses glucose and fructose (but not galactose) is known as Crabtree effect. Addition of glucose to cell culture induces immediate transition to anaerobic metabolism, lactate production *(Burd et al. 2001)* and, mainly, pronounced decrease of cellular respiratory rate. Mechanism of Crabtree effect is mostly unresolved. Up to date, there is no clear explanation or molecular mechanism proving, that during Crabtree effect, direct inhibition of mitochondrial respiratory system occurs; or if there is a regulatory mechanism at the level of substrate administration between aerobic and anaerobic metabolic pathways; or triggering of cellular signalization causing activation/inactivation of target enzymes involved.

Elevated glucose intermediates, particularly FBP, were suggested to affect respiratory complexes and respiration in yeast cells (Diaz-Ruiz et al. 2008). Another report describes the effect of extracellular pH on respiration of melanoma cells *(Burd et al. 2001)*. Melanoma cells, which normally exhibit pronounced Crabtree effect, adapted to growth in low pH (6.7) exhibit glucose-stimulated increase of respiratory rate and decrease of lactate production *(Burd et al. 2001)*. This is an important finding demonstrating that within a tumor, multiple regions of distinct metabolic activity may exist dependent on metabolite concentration, in this case lactate. By reversal of Crabtree effect, cells which grow in already acidic environment overcome further acidification and protect themselves by potentially harmful consequences of lactate production.

1.5. Hypoxia

Administration of oxygen to single cells within tissue is held by microvascular system. Oxygenation of well perfused tissues ranges of 30 – 60 mmHg. Homeostasis between oxygen demand of metabolically active tissue, oxygen delivery and release is regulated by blood flow variations, pH, and diffusion flux from the microvessels. On the contrary, tumor vasculature is often structurally and functionally impaired and provides an inadequate oxygen supply.

Mitochondrial respiration rate is independent of oxygen when oxygen is abundant, but declines with declining oxygen pressure in low oxygen range *(Gnaiger et al. 1995)*. Respiration in low

oxygen pressure, as described by Chance *(Chance 1965)*, is a hyperbolic function, with P_{50} ranging from isolated mitochondria to intact cells. So, as a primary regulatory kinetic mechanism, oxygen flux through mitochondrial respiratory system is imposed by oxygen itself. Secondary adaptive changes to low oxygen pressure include complex redox-, cellular signalization and protein expression changes.

Typical feature of certain cancer cells is high lactate production, resulting in cytosolic and extracellular acidification. Activation of glycolysis provides sufficient ATP levels under the conditions of increased energetic demand in hypoxia or in highly proliferative tissues. Expression of LDH is controlled by HIF pathway *(Ebert and Bunn 1998)*. Primarily, increase of LDH expression is an adaptation to low oxygen. Obviously, there is tight interconnection between glycolysis and oxidative phosphorylation, since LDH inhibition results in increased respiration *(Fantin et al. 2006)* and vice versa.

Also an active inhibition of oxidative metabolism seems to be a part of adaptation to hypoxia. Another important target of HIF is pyruvate-dehydrogenase kinase 1 (PDK-1). Mitochondrial pyruvate conversion to acetyl-coenzyme A (Ac-CoA) by mitochondrial pyruvate dehydrogenase complex (PDH) is attenuated by pyruvate-dehydrogenase kinase, another important target of HIF pathway. PDK-1 induced inactivation of PDH in hypoxia effectively decreases mitochondrial oxygen consumption *(Papandreou et al. 2006)*. Inhibition of oxidation of pyruvate in hypoxia provides growth advantage, since it makes pyruvate available for LDH. Pyruvate to lactate conversion and glycolytic ATP production is therefore accelerated.

1.6. Role of proliferation rate in cancer metabolic remodelling

To maintain tumorigenic potential, sustained high proliferation rate of cancer cells during invasive growth is necessary. Proliferation rate is, however, very demanding on biomass formation and requires appropriate metabolic adaptations to provide constant supply of proteins, lipids and de novo synthesis of purines and pyrimidines as well as ribose for nucleic acid synthesis to form new cells. Glucose has at least two possible fates within the cell: it may be oxidized to produce cellular bioenergy or it may be converted into other macromolecules to support cellular biosynthesis. Reports of metabolic studies of human glioblastoma cells indicate, that despite low respiration, constant pyruvate flux through PDH is maintained *(DeBerardinis et al. 2007)*. Pyruvate, product of glucose metabolism, imported into mitochondria, is major precursor of Ac-CoA but also the citrate for FA synthesis, particularly phospholipids, essential for cell growth. In the same study, the role of glutamine metabolism was highlighted. By glutamine metabolism, NADPH pool is maintained for cellular biosynthetic pathways, and recovers low NADPH production by PPP. Major contribution of glutaminolysis to lipid synthesis

is production of oxaloacetate or malate, which replenish TCA cycle by cataplerotic reactions and along with glucose-derived Ac-CoA participate in citrate synthesis. Utilization of two distict pathways, glycolysis and glutaminolysis, provides glioblastoma cells ability to generate biosynthetic precursors for multiple catabolic pathways, form biomass, reflected in growth advantage over non-transformed cells. New lipid biomass might be derived from the conversion of other macromolecules to lipids or from the utilization of pre-existing extracellular lipids. Proliferative cells as well as cancer cells, however, utilize preferably de-novo synthesis of FA *(Jackowski et al. 2000)*. Accordingly, major cellular lipid precursor remained glucose over glutamine *(DeBerardinis et al. 2007)* based on detection of radioactive tracer.

Depression of respiration in cancer cells in general can be well explained by lack of substrate due to the pyruvate shunt towards fermentation. These glucose-derived carbons, when incorporated into citrate, may either be oxidized via the TCA cycle or exported from mitochondria (cataplerotic flux). It was reported, that depression of respiration in proliferating cells in normoxia was caused by lack of substrate for OXPHOS due to the elevated citrate efflux from mitochondria (during glutaminolysis) to maintain high rate of FA synthesis *(DeBerardinis et al. 2007; Bauer et al. 2005)*. In this case, reduction of respiration is secondary to metabolic activities needed for biosynthesis of proliferating cells. Once citrate exported of mitochondria, cytosolic citrate is processed by ATP citrate lyase (ACL) to produce cytosolic Ac-CoA and regenerate oxaloacetate. Cytosolic Ac-CoA is the requisite building block for all endogenous synthesis of acyl groups and sterols. ACL expression/inhibition likely regulate formation of cytosolic Ac-CoA, governs the direction of citrate towards oxidation by TCA cycle, and mitochondrial oxygen consumption *(Bauer et al. 2005)*. Combination of glucose presence and ACL overexpression provides tumor cells an advantage to proliferate extensively *(Hatzivassiliou et al. 2005)*. Upon interruption of its high glucose-to-lipid flux, factors in the in vivo tumor microenvironment may allow a tumor cell to initiate adaptive responses, such as differentiation, in order to maintain its survival and compensate for the growth rate. Disrupting citrate transport offers the possibility to treatment strategies *(Mashima et al. 2009)* by suppressing of proliferation rate of tumor cells.

1.7. Metabolism of glutamine in cancer cells, glucose deprivation

Although metabolism of cancer cells is strongly dependent on glucose, depletion of glucose can occur in solid tumors; therefore, cancer cells possess alternative metabolic adaptations to overcome glucose deprivation. Among these, utilization of fatty acids (FA) can be used as metabolic substrate, or alternatively, utilization of glutamine to support cancer growth. Some tumor-derived cells have been reported to grow in glucose-free medium supplemented with

amino acids *(Helmlinger et al. 2002)*, which was a result of altered channeling of amino acids into metabolic pathways, namely glutamine, as compared to normal cells and tissues. In HeLa cells, although only 5% of metabolized glucose entering the TCA cycle, significant oxidative ATP production is maintained owing to glutamine metabolism *(Reitzer et al. 1979)*. Glutaminolysis is the alternative pathway utilized to replenish TCA cycle and produce ATP by oxidative metabolism even in the presence of glucose *(Reitzer et al. 1979)*. Elevated glutamine-dependent metabolism was observed in various types of tumors (Matés et al.). Glutamine which entered Krebs cycle (in form of KG) does not have to be metabolized completely, but readily exported of mitochondria as malate and catalysed by the cytoplasmic malic enzyme (ME) to pyruvate. This reaction results in NADPH production, largely induced along with glutaminolysis. Pyruvate formed by the reaction of malic enzyme (ME) contributes to the lactate production *(Reitzer et al. 1979; DeBerardinis et al. 2007)*. Indeed, 60% of glutamine was observed to be metabolized to lactate in human glioblastoma cells *(DeBerardinis et al. 2007)*. Alternatively, citrate efflux supports the FA synthesis. Bi-product of FA synthesis OAA is reduced to malate via cytosolic malate dehydrogenase and is imported to mitochondria via malate/α-KG exchanger. Reaction of malate dehydrogenase is driven if excess NADH existing in cytoplasm (as occurs with high rate of aerobic glycolysis). Increased lipid metabolism, particularly phospholipids synthesis, was observed as a response to activation of glutaminolysis through transcriptional regulation of glutamine transporters and glutaminolytic enzymes induced by Myc oncogene *(Wise et al. 2008)*. If complete TCA takes place, malate imported to mitochondria can replenish TCA cycle. However, due to the increased activity of mitochondrial ME *(Moreno-Sánchez et al. 2009)*, malate is preferably metabolized to pyruvate. Pyruvate along with glutamine can support full TCA cycle activity.

Glutaminolysis can in fact compensate for cellular ATP, pyruvate, lactate and NADPH pool, normally supplied by glucose metabolism via PPP. Requirement for glutaminolysis is, however, aerobic conditions. Therefore, it is possible that some cells within tumor adapt to current substrate by reprogramming of energy metabolism according to the actual exogenous substrate. Furthermore, combination of substrate availability therefore notably influence metabolism of cancer cells.

1.8. Mitochondrial uncoupling proteins expression

Among members of uncoupling protein subfamily, eight distinct isoforms can be recognized which form one branch of mitochondrial anion carrier gene family, containing 46 members in mammals *(Hanák and Jezek 2001; Ježek and Urbánková 2000)*. In mammals, isoforms UCP1 to UCP5 are recognized and three other isoforms (originally termed PUMPn) exist in plants.

More than decade of studies did not answer the basic question of its function in mitochondria and its physiological roles, neither demonstrated in indisputable manner exemplar situations under which such roles might be executed. The originally suggested functional roles were later disputed such as for attenuation of reactive oxygen species (ROS) formation *(Nedergaard and Cannon 2003; Cannon et al. 2006; Fisler and Warden 2006; Krauss et al. 2005; Mattiasson and Sullivan 2006)* since mild uncoupling is the principal regulator of oxidative stress *(Dlasková et al. 2008a; Plecitá-Hlavatá et al.)*, besides ROS protection has a wide implications to cell physiology and pathology. Among other physiologically acceptable roles of UCPS, regulation of glucose-stimulated insulin secretion (GSIS) *(Zhang et al. 2001; Joseph et al. 2004)*, and regulation of mitochondrial Ca^{2+} handling *(Trenker et al. 2007; Wu et al. 2009; Brookes et al. 2008)*, chemoresistnce of tumors *(Derdak et al. 2008)*, neuroprotection *(Mattiasson and Sullivan 2006)*, and atherosclerosis *(Blanc et al. 2003)* have been proposed.

UCP1, originally ascribed exclusively to brown adipose tissue, has been reported recently in thymocytes *(Carroll et al. 2005; Adams et al. 2008a; Adams et al. 2008b)*, pancreatic β-cells *(Sale et al. 2007)*, thymus *(Carroll et al. 2005; Frontini et al. 2007; Carroll et al. 2004)*, skin *(Mori et al. 2008)*, and brain *(Jastroch et al. 2007)*. UCP2 is considered to be expressed ubiquitously in mammalian tissues *(Fleury et al. 1997; Gimeno et al. 1997; Lengacher et al. 2004)*. In contrast, UCP3 mRNA has been detected in northern blots of human skeletal muscle and in rodent skeletal muscle, heart, and BAT originally *(Boss et al. 1997; Vidal-Puig et al. 1997)*. The original reports for UCP5 and UCP4 mentioned their high expression in the central nervous system *(Mao et al. 1999; Sanchis et al. 1998)*. Northern blotting also detected UCP5 mRNA in both mouse and human heart, kidney *(Kim-Han et al. 2001; Sanchis et al. 1998; Yu et al. 2000)* and skeletal muscle *(Lengacher et al. 2004; Sanchis et al. 1998; Yu et al. 2000)*. However, Sanchis et al. (1998) found even wider distribution of UCP5 mRNA, in the rat, mouse, and human gut, lung, testis, uterus, spleen, and white and brown adipose tissue. Additional findings were reported for the human prostate, pancreas, adrenal medulla and cortex, thyroid gland and liver, as well as for the mouse liver (but not spleen), lung, and skeletal muscle *(Yu et al. 2000)*. Using quantitative RT-PCR with TaqMan probes, *Lengacher et al.* (2004) found not only UCP4 and UCP5 mRNA in the mouse brain cortex (together with a lower amount of UCP2 transcript), but also ~100 times lower levels of UCP3 and UCP1 mRNA. In BAT and skeletal muscle, *Lengacher et al.* (2004) also identified all five UCP mRNAs, with the UCP1 mRNA level in BAT being ~100-fold higher than all other isoforms, and with UCP3 mRNA level being at least 10-fold higher in skeletal muscle. It is unclear, however, whether the minute levels of UCP mRNAs become translated into mature proteins. If indeed they were translated, one must re-evaluate the molecular physiology of UCPs based on their tissue distribution in light of

findings such as UCP1 mRNA in human pancreatic β-cells *(Sale et al. 2007)*, UCP5 in endocrine cells *(Ho et al. 2005; Ho et al. 2006)*, UCP4 mRNA in preadipocytes *(Zhang et al. 2006)*, or of the emerging role of UCP4 and UCP5 in brain pathologies *(Liu et al. 2006; Chan et al. 2006; Nakase et al. 2007; Naudí et al. 2007)*. Observed disparity between the abundance of certain mRNAs and their corresponding protein products has been attributed to both translational down-regulation *(Pecqueur et al. 2001; Hurtaud et al. 2006)* and up-regulation *(Hurtaud et al. 2007)*.

1.9. CIDE proteins and their role in apoptosis

DFF is important effector of apoptosis performing the final step of apoptosis – DNA fragmentation/chromatin condensation. It is a heteromultimeric protein *(Lechardeur et al. 2005)* that consists of the 40-kDa caspase-3-activated nuclease (DFF40, CAD), and its 45-kDa inhibitor (DFF45, inhibitor of CAD or ICAD). Cleavage of DFF complex by caspase-3 liberates the functional nuclease thus promoting the internucleosomal DNA fragmentation *(Inohara et al. 1999; Bayascas et al. 2004; Erdtmann et al. 2003)*. CIDEs are related to N terminal of both subunits of the DFF. There are three members of so-called cell death-inducing DFF45 (DNA fragmentation factor)-like effector (CIDE) protein family described: CIDE-A *(Inohara et al. 1998; Zhou et al. 2003)*, CIDE-B *(Inohara et al. 1998; Lugovskoy et al. 1999; Chen et al. 2000)* and CIDE-3/FSP27 *(Liang et al. 2003)*. CIDEs are small proteins (~25kDa) expressed in two isoforms arising from alternative splicing *(Liang et al. 2003)*.

Since their identification, studies of CIDE proteins were concentrated on tracing possible connection with control of apoptosis induction or progression. Overexpression of CIDE-A and CIDE-B results in induction of apoptosis and DNA fragmentation *(Inohara et al. 1998)*. Importantly, CIDE-induced apoptosis was not inhibited by caspase inhibitors, but was inhibited by DFF45. Nuclease function of CIDE-A or CIDE-B proteins, such as for EndoG, was not reported. Instead, action of CIDE-A and CIDE-B proteins, is more likely connected to DFF40 and DFF45 and provides a possibility of regulation of DFF activation by domain interactions, and independently of caspases.

Model of CIDE-related activation of apoptosis was proposed as follows. The CIDE-N domain, common with DFF, can bind to the homologous domain on DFF45 opposing its inhibitory effect on DFF40 *(Lugovskoy et al. 1999)*, FIG C-2. However, it is not clear whether CIDE has higher affinity for DFF45 than DFF40 and is thus able to release DFF40 from the complex allowing it to exert its nuclease activity. One should also bear in mind other regulatory mechanisms of apoptosis, such as that DFF45 is synthetised in excess to DFF40 ensuring fine tuning of DFF activation and decreasing the probability of CIDE-N buffering by CIDE proteins.

2. AIMS AND HYPOTHESES

Adjustments of metabolic energy pathways of cancer cells are reflected in elevated vitality and high probability to survive detrimental conditions occurring in solid tumors, such as hypoxia and glucose deprivation. Growing body of evidence indicate large complexity in alterations of energy pathways and its modulation by tumor microenvironment in cancer cells. *Main objective of this thesis was to analyze the strategies that cancer cells utilize in order to survive harsh conditions, namely glucose and oxygen limitation, similar to those present in solid tumors.* The biological question under investigation is of particular importance for a better understanding of cancer biology and energy metabolism regulation. *Thus, the aim of my thesis was to analyse the impact of glucose deprivation and oxygen limitation on the bioenergetic properties of cancer cells in comparison with non-cancer cells. It was the intention to determine the capacity and the functioning level of the mitochondrial oxidative phosphorylation in breast cancer cells. Then, we followed whether microenvironmental metabolic stresses (i.e glucose and/or oxygen limitation) could reshape this system to permit cell survival.* Other part of work deals with quantification of novel UCPs expression. *The aim of this work was to present the quantification of UCPs transcripts in rat and mouse tissues brain, heart, kidney, liver, spleen, skeletal muscle and white adipose tissue*. Historically, UCP2 has been presented as a protein with wide expression pattern throughout the body tissues, since UCP3, UCP4 and UCP5 exhibited more specific expression pattern. Last part of work concentrates on human CIDEa protein and its role in apoptosis regulation. According to the proposed model, CIDE proteins binds to the DFF-45 protein which is an inhibitor of DFF-40 nuclease thus activate apoptotic DNA fragmentation independently of apoptosis. Prerequiresite of this model is localization of CIDE proteins into the cytosolic or nuclear fraction. The aim was *to explore cellular localization of CIDEa proteins under apoptotic conditions to reveal possible cellular migration of this protein thus attempted to determine its function in apoptosis.*

In summary, aims were designed as follows:

- To analyze the impact of glucose and oxygen limitation on the overall survival of cancer cells and corresponding normal cells, mainly their bioenergetic profile and mitochondrial features.
- To to present the quantification of mitochondrial uncoupling proteins transcripts in rat and mouse tissues brain, heart, kidney, liver, spleen, skeletal muscle and white adipose tissue.
- To elucidate cellular function of CIDEA protein, its possible migration between cellular compartments and organelles and thus attempt to determine its function in apoptosis.

3. METHODS

In this thesis, several principial methods were used:

- cell cultures – different culturing conditions;
- high resolution respirometry and oxygen kinetics;
- real-time PCR;
- western-blotting;
- molecular biology tools: expression plasmids preparation, protein expression, deletion mutants of CIDEa protein preparation.

4. RESULTS

4.1. Glucose deprivation and Crabtree effect

Cells were grown in Glc and Gln/GAL medium for prolonged time (minimum of 4 days) and cell growth was followed. Cultivation in glucose-deprived conditions caused significant decrease in growth rate, resulting in increase of doubling time of the culture both for cancer and normal cells. Doubling time of HTB-125 cells in Glc medium was 34±1.5 hours, and 28±1.3 hours for HTB-126 cells. Cultivations in the Gln/GAL medium resulted in augmentation of doubling time (slower growth) to 47±2.1 and 39±1.1 hours for HTB-125 and HTB-126 cells, respectively.

To follow the functional adaptation of the mitochondrial oxidative phosphorylation of cells grown in glucose or glucose-deprived medium, a high-resolution respirometry was used. Respiration of intact cells was measured. We have compared the respiratory flux values (routine respiration of intact cells supported by intrinsic substrates) measured i) in glucose medium (Glc), ii) after the glucose removal (instant replacement by Gln/GAL medium, Gal0), and iii) in cells adapted to the growth in the glucose-deprived medium (GAL 4, such adaptation occurs after 4 days of growth in the glucose deprived-medium). The growth under glucose-deprived conditions (minimum of 4 days) leads to a large increase in the respiratory flux of cancer cells (90.7±4.8 pmol $O_{2*}s^{-1}*10^{-6}$cells) compared to Glc cells (24±4.8 pmol $O_{2*}s^{-1}*10^{-6}$cells). No significant increase was observed in non-cancer HTB-125 cells (41.4±1.6 in Glc, 49.9±4.4 in the Gln/GAL medium, respectively).

The removal of glucose and its replacement by Gln/GAL medium (Gal0) led to a two-fold increase in cell respiration of cancer cells HTB-126 (40±4.8 pmol $O_{2*}s^{-1}*10^{-6}$cells), whereas no significant change was observed in the non-cancer cells. The difference between Glc and GAL0 group might be a result of the inhibitory effect of glucose exerted on mitochondrial respiration. The increased respiratory flux of Gal0 group was attributed to the removal of Crabtree effect.

For cancer and control cells, we have determined the oxygen pressure at 50 % of maximum flux (P_{50}), which gives a measure of the apparent cellular affinity to oxygen. P_{50} can be determined by high-resolution respirometry by plotting the flux value as a function of the p_{O2} and fitting with a theoretical hyperbolic curve. The P_{50} values revealed higher P_{50} values in the cancer cells, in comparison to the non-cancer cells. A slight decrease of P_{50} values observed in cells grown under high glucose as compared to the glucose-deprived medium (GAL4) indicated a lower apparent affinity of the cellular respiratory system to oxygen. Besides characterization of the cellular

response to hypoxia, P_{50} is an indicator of the activity of electron transport chain (ETC), particularly COX turnover rate. Increase of P_{50} value of HTB-126 GAL4 group compared to HTB-126 Glc is associated with an increase of activity and turnover rate of the respiratory chain components upon sustained growth in glucose-limiting conditions. However, slight increase of P_{50} does not correspond to robust increase of respiratory rate (4-fold increase of GAL4 compared to Glc) that presumes large increase of P_{50}. Although the respiratory flux was significantly higher for the glucose-deprived cells, P_{50} values differed only by 10%, indicating higher, but still comparative metabolic state of both Glc and GAL4 groups.

Determination of respiratory activity of respiratory complexes

Using specific inhibitors of ETC, impact of respiratory complexes I and II to the overall respiratory activity was analyzed. The cellular endogenous flux (routine respiration, corresponding to the *in situ* state of mitochondrial respiration) is a result of oxidation of endogenous substrates (NADH and $FADH_2$ derived from cellular catabolic reactions of glucose and lipids) by ETC. Routine respiration is a function of ATP demand and substrate availability, which could be considerably limited. Using inhibitors rotenone and subsequently antimycin A, we could estimate complex I and complex II respiratory activity. We have observed that complex II was involved exclusively in HTB-126 GAL cells, although only by 20%, since rotenone inhibited respiration completely, usually.

Therefore, we have investigated functionality of complex II using respiration of permeabilized cells with exogenous respiratory substrates of complex I (glutamate and malate) and complex II (succinate), in the presence of ADP. We have shown that complex II is functional both in HTB-126 Glc and GAL cells, as respiration was sensitive to addition of complex II succinate.

Crabtree effect

Respiration of cancer cells was observed to be widely inhibited by glucose addition. Crabtree effect occurred in HTB-126 cells. During transition to low-respiratory state, or to the high-respiratory state, respectively, turnover of ETC enzymes should be decreased or increased, respectively, associated with concomitant P_{50} changes. Glucose addition and removal, however, did not result in alteration of cellular P_{50}. This suggest, that mitochondrial enzymes of respiratory chain could be inhibited when glucose present. To confirm possible inhibition at the level of mitochondrial respiratory chain, respiration with uncoupler was performed. Maximal respiratory

capacity with uncoupler of HTB-126 Glc 39±3.7 pmol $O_{2*}s^{-1}{}_{*}10^{-6}$cells. Removal of glucose resulted in increased flux to the level comparable to the maximal capacity of HTB-126 Glc. Addition of uncoupler stimulated respiration to even higher extent, confirming that glucose presence inhibits the respiration at the level of respiratory chain enzymes. Accordingly, glucose addition resulted in inhibition of endogenous respiratory flux as well as uncoupled flux. The data present in this section indicate an inhibition of electron transport through complexes I or II when glucose is present. The removal of glucose causes the extension of ETC capacity. The exact nature of this effect requires further investigations.

Expression of OXPHOS proteins

The expression levels of respiratory chain proteins (subunits of complex I, complex II and complex IV) were measured by westernblot on cell lysates prepared from HTB125 and HTB126 cells, grown in glucose medium or glucose deprived medium for 4 days. Densitometric analysis indicates the upregulation of protein expression with a mean factor of 1.26 ± 0.098, 1.88 ± 0.48 and 2.39± 0.15 for CI, CIV and CI in non-cancer cell grown in absence of glucose, respectively. In cancer cells, this increase was more pronounced and the mean factor value was of 1.28 ± 0.01, 4.17 ± 0.34 and 5.97 ± 1.7 for CI, CIV and CI, respectively. These data evidence a stronger induction of OXPHOS proteins expression in glucose-deprived medium of cancer cells, suggesting activation of biogenesis upon glucose-deprivation in cancer cells not in normal cells.

4.2. Hypoxia

The effect of hypoxia was studied while cells were incubated at normoxia, or at hypoxia (1% oxygen) during 1 or 6 days, and cell viability was assessed using the neutral red assay during the exponential phase of growth in glucose or galactose-glutamine medium. In HTB-125 non-cancer cells grown in glucose medium, hypoxia induced a decrease in cell viability at day 1 (20 ± 14 % reduction), and no significant change at day 6. Conversely, in cancer cells (HTB-126), 6 days of hypoxia resulted in a large increase in cell viability (54 ± 6 % increase). In glucose-deprived medium, 6 days of hypoxia induced a significant decrease of cell viability both in HTB-125 cells (51 ± 8 % reduction after 6 days), or in cancer HTB-126 (all the cells died). This suggests that 6 days of growth at 1% O_2 hypoxia exerts opposite effects on cancer cells viability, depending on the presence or absence of glucose in the culture medium.

Respiration

The endogenous respiration of HTB-125 and HTB-126 cells grown in glucose or glucose-deprived medium was measured after 4 days in normoxia or 1 % hypoxia, as was the pH value of the cell culture medium. In non-cancer cells grown in glucose medium, 1 % O_2 hypoxia significantly enhanced cell respiration by 34 % after 6 days. This was also observed in glucose deprived medium (26 % increase). Conversely, in cancer cells 1 % O_2 hypoxia led to a reduction of cell respiration both in glucose medium or glucose-deprived medium (36 % and 24 % reduction, respectively). The pH measurement revealed a significant medium acidification in cancer cells grown under aglycemia and hypoxia. This argues for a different sensitivity and a variable response of cell energy metabolism toward hypoxia, in cancer cells versus the non-cancer counterpart.

Expression of OXPHOS proteins in hypoxia

To match alterations of cell viability and respiration with OXPHOS proteins expression after hypoxia exposure, western-blot analysis was performed. For HTB-126 Glc cells, we have obtained pronounced decrease of all respiratory complexes as a result of hypoxia exposure. Concersely, no significant decrease was obtained in HTB-126 GAL cells. These results suggest that mitochondrial OXPHOS proteins are widely decreased in response to hypoxic exposure, resulting in decrease of respiratory capacity. This adaptation is accompanied with increased viability of cancer cells when glucose is present.

4.3. Absolute quantifications of UCPn transcripts

We have confirmed the presence of UCP2 mRNA in all eight rat tissues studied (FIG). The absolute amount of UCP2 mRNA (in pg per 10 ng of total isolated mRNA) decreased in the order of spleen > heart > lung > WAT > brain > skeletal muscle > kidney > liver, spanning a 30-fold difference in UCP2 transcript content. Only in the rat spleen and lung were the UCP2 transcript levels of the same order of magnitude as the control GAPDH transcript levels. With the exception of the heart UCP2 mRNA, a similar pattern was found for mouse tissues.

As expected *(Boss et al. 1997; Vidal-Puig et al. 1997)*, we detected the highest UCP3 mRNA levels in rat and mouse skeletal muscle and intermediate levels in mouse heart, the latter being of the same order of magnitude as the UCP2 lung transcript levels. In contrast to the very high UCP2 mRNA levels found in the rat heart, the UCP3 mRNA was much less abundant.

The absolute mRNA levels of the UCP4 isoform in rat tissues were generally among the lowest determined. There were by up to three orders of magnitude lower than the UCP2 mRNA levels. As expected (*Mao et al. 1999*), the maximum UCP4 mRNA level was in the brain, but still two orders of magnitude lower than the UCP2 mRNA level. In turn, UCP4 transcript abundance in the rat brain (similarly for UCP5, see below) was similar to that of UCP2 mRNA in the mouse lung. The mouse skeletal muscle UCP4 transcript level exceeded that of UCP2. Similarly, mouse WAT contained nearly equivalent levels (~0.05 pg per 10 ng of total isolated mRNA) of UCP2, UCP3, and UCP4 transcripts. The UCP4 transcript level was not increased in the brain of the $UCP2^{-/-}$ mice compared with wild-type mice.

Similar to the rat UCP3 and UCP4 mRNAs, rat UCP5 mRNA was primarily expressed in a single tissue, namely brain. Whereas in the case of rat UCP3 such an apparent exclusivity was found in the skeletal muscle, in the case of rat UCP5 it was the brain. In other tissues the levels of UCP5 mRNA (~10–3 pg per 10 ng of total isolated mRNA) transcript levels of UCP5 were similar to the UCP3 levels. The rat UCP5 mRNA levels were still at least three-fold higher than those of UCP4. The tissue distribution of mouse UCP5 mRNA expression was somewhat more heterogeneous compared with the other UCPs, although UCP5 mRNA levels in brain were highest among the tested tissues. Notably, ~0.5 pg per 10 ng of total isolated mRNA was found for the UCP5 and UCP4 transcripts in the mouse brain, as well as for UCP2 mRNA in mouse lung and UCP3 mRNA in mouse skeletal muscle. The mouse heart UCP5 mRNA level was equivalent to that of UCP3. UCP5 mRNA in WAT and spleen was even more abundant than UCP3 mRNAs in these tissues and UCP2 mRNA in WAT. Although only at levels of ~0.05 pg per 10 ng of total isolated mRNA, UCP5 was the predominant isoform in the mouse liver and kidney. The UCP5 transcript was not elevated in the brain of $UCP2^{-/-}$mice.

4.4. Cellular localization of CIDEa and its redistribution upon apoptosis induction

In this work, we have localized CIDEa protein to mitochondria under physiological conditions by various imaging techniques and confirmed a specific role of CIDE-C domain in mitochondrial localization of CIDEa protein. When treated with valinomycin and camptothecin, CIDEa redistribution is more apparent and corresponds with the quantification of CIDEa-positive cells displaying at least some nuclear localization of CIDEa. Heterogeneous cell population was present in our experiments with variable percentage of cells undergoing apoptosis. Pretreatment of cells with pan caspase inhibitor z-VAD-fmk did not significantly alter the percentage of CIDEa positive cells displaying apoptotic morphology, i.e. cells displaying

shrinkage and/or nuclear fragmentation, thus demonstrating the caspase-independence of the process. On the contrary, the percentage of CIDEa positive/apoptotic cells insamples treated with valinomycin was significantly higher than those treated with DMSO (78 vs. 40%) thus supporting the link between redistribution of CIDEa and apoptosis.

5. DISCUSSION

Non-canonical bioenergetics concerns with those physiological and pathophysiological situations under which ATP synthesis is suppressed. This thesis brings an outcome of three types of studies within the field of the non-canonical bioenergetics, investigating specific bioenergetic phenotypes of cancer cells, on one hand; and a role of mitochondrial uncoupling proteins as deduced from their transcript distribution in various tissues and organs; plus a role of a novel and likely pro-apoptotic factor CIDEa in mitochondria. In this work, we have shown, that OXPHOS composition, content and performance depends largely on substrate availability and cell type. Despite a wide belief of the mitochondrial aberration in cancer cells, we have demonstrated that mitochondrial respiration is intact and fully competent in HTB-126 cells. Cancer and normal cells void of glucose are able to maintain cell proliferation under the normoxic conditions albeit with considerable loss of proliferation rate. Respiration of HTB-126 cells grown under standard high-glucose conditions is four-times lower than after the adaptation to Gln/GAL medium, short of glucose.

We have investigated why the respiration is low in the HTB-126 Glc cells. We can only predict that sustained glucose presence possesses a stimulatory effect on glucose metabolism and/or inhibitory effect on oxidative metabolism. We assumed that in HTB-126 cells, glucose supports the cells growth along with the ATP production. In order to support the cell growth, FA synthesis should be enhanced to support membrane synthesis. The major precursor of FA synthesis is citrate, synthesized in TCA cycle. TCA cycle does not have to be necessarily intact *(Owen et al. 2002)*. It has been frequently recognized that truncation of TCA cycle might occur in number of cell types and serves to synthetize number of metabolic precursors for anabolic processes (cataplerotic flux). When glucose serves as a chief energy substrate, remaining carbons that are not deviated to lactate synthesis, enter a truncated TCA cycle where citrate is preferentially extruded to the cytosol and feeds the fatty acids and sterol synthesis. We have shown, that in intact cancer cells, complex II is not involved in respiration despite the obvious functionality with respiratory substrate succinate. The citrate extrusion of mitochondria would explain the low respiratory flux of HTB-126 Glc cells with the exclusive complex I electron input. Such organization of metabolic pathways and metabolite sorting would theoretically support the high cell growth rate of cancer cells grown in high-glucose medium. Similarly, in HTB-126 GAL cells, complex II participate in respiration of intact cells by minor part. We have concluded that the substrate flux through TCA cycle is probably compromised in intact cells with regard to the succinate-related respiration in permeabilized cells. This phenomenon can be explained on the

basis of cell-growth support, as reported previously *(DeBerardinis et al. 2007)*. In this case, metabolites of TCA cycle are rather utilized as anabolic precursors of FA rather than for completion of TCA cycle. Thus the citrate efflux of mitochondria would depress the substrate flow through succinate-dehydrogenase complex and explain the absent flux through complex II. NADH reoxidation was detected as complex I respiration represents the major part of respiratory activity of HTB-126 GAL intact cells. NADH production in TCA cycle cannot operate without a source of Ac-CoA. Pyruvate as a precursor of Ac-CoA can be gained from culturing media, and synthesized by cytoplasmic or mitochondrial malic enzyme from malate.

We hypothesized, that presence of glucose has an inhibitory effect on respiration additionally to being a preferred energy source and FA precursor. Such an effect has already been described as Crabtree effect. This is demonstrated by an immediate increase of respiration upon removal of glucose medium and its replacement by Gln/GAL medium. In this case, we have obtained a 40% increase of respiratory rate due to the glucose removal. This 40% can be attributed to the removal of Crabtree effect. The remaining increase of respiration is attributed to more general adaptation that cells undergo in order to survive glucose deprivation. This adaptation occurs, however, on the expense of cell growth rate, which is considerably retarded under the glucose-limiting conditions. However, elevated respiratory capacity of HTB-126 GAL cells cannot be only attributed to removal of glucose inhibitory effect. The difference of respiratory capacity between of Glc and Gal0 group is attributed to removal of glucose inhibitory effect, since increased flux of GAL4 as compared to Gal0 group must have other origin. We proposed, that mitochondrial biogenesis was induced during adaptation to glucose starvation based on the elevated OXPHOS protein levels.

Cancer cells HTB-126 GAL were unable to survive under hypoxic conditions on contrary to HTB-126 Glc cells. This is interesting difference, which seems trivial at the first sight. Mitochondrial respiration, mitochondrial P_{50} and OXPHOS proteins content and did not change after hypoxia exposure. All data predicts that the decrease of mitochondrial biogenesis did not occur. Control cells under the same conditions were able to survive hypoxic conditions, only at the expense of decrease viability by 20%. We have also observed a decrease of mitochondrial content by microscopy and western-blot. In cancer cells, inhibited biogenesis pathways in hypoxia have been reported previously *(Zhang et al. 2007)* and we support the respective hypothesis predicting these changes to occur. On contrary, no decrease of OXPHOS components has been observed in glucose-deprived medium. It remains to be determined, if HIF stabilization was affected due to the lack of glucose. Similarly, it is to be understood if mitochondrial

degradation is the crucial step in hypoxic adaptations of cancer cells along with induction of glycolysis. In this case, suppression of mitochondrial degradation could be the crucial deficit for survival of cancer cells in 1% oxygen.

In conclusion, we hypothesize, that inhibition of biogenesis might be of equal importance in survival of hypoxia than induction of glycolysis in cancer cells. Cancer cells deprived of glucose can not survive hypoxic exposure, since inhibition of respiration is infeasible upon glucose deprivation. Mitochondria of HTB-126 GAL cells are not aberrant in glucose limiting conditions, but not able to provide energy to maintain cell growth under the hypoxia. However, what seem disadvantageous for under certain conditions can possibly be advantageous for cancer cells under different condition beyond the scope of this study, such as invasion to the glucose-limiting areas. Under our experimental conditions, glucose deficiency seems to be superior to oxygen deprivation.

In second project, we have revealed wide expression pattern of individual UCP isoform mRNA levels that varied by up to four orders of magnitude in rat and mouse tissues, with highest expression of UCP2 transcript in the rat spleen. The finding that UCP2 transcript abundance was highest in the spleen points to important physiological role of UCP2 in macrophages *(Arsenijevic et al. 2000; Giardina et al. 2008)* and other white blood cell types, resident in the spleen. Similarly, the second highest UCP2 transcript amount, found in the rat and mouse lung, may reflect its predominating expression in the alveolar macrophages.

Attempting at least to find the order of magnitude proportionality between the measured levels of UCP mRNAs and their corresponding protein products, we can compare data for mouse spleen. These data show that UCP2 accounts for ~0.03% of all mitochondrial proteins (*Pecqueur et al. 2001*). Compare this to our finding of 0.002% for UCP2 transcript abundance . Considering on average 10,000 transcribed genes as 100%, these data would match only, if approximately each tenth transcribed protein in general was mitochondrial. The reported mouse lung UCP2 protein level (~0.002 % of rat mitochondrial proteins, *Pecqueur et al. 2001*) seems to correlate with a rather high estimated transcript abundance of 0.004%. Also, our [3H]GTP binding study reflecting UCP2 protein amounts (*Žáčková et al. 2003*) correlated better with the rat UCP2 transcript quantification, indicating rather high amounts of UCP2 protein in rat lung mitochondria, and 10-fold lower levels in rat liver mitochondria (*Žáčková et al. 2003*). However, specific quantification of UCP protein level and to distinct the specific isoform with an antibody is impossible to our knowledge.

We have shown that the tissue-specific expression pattern of individual isoforms is quite distinct between rat and mouse and that the levels of mouse UCP4 and UCP5 transcripts, previously considered to be very low, are in fact quite high. The question, what transcript amount tells us about the protein level and if the low transcription of UCPs isoforms really results in translation and maturation of the mitochondrial protein, is a matter of debate. The finding that numerous tissues express more than one UCP isoform sheds new light on previous studies in mice in which one isoform, UCP2 or UCP3, was ablated. Their reported interpretations might be yet inconclusive due to the possible expression of other UCP isoforms, if one considers the possibility of functional redundancy among UCPs. However, in attempt to see any compensatory increase of other UCP isoform after UCP2 ablation, no difference has been found.

In another project, we determined mitochondrial localization of CIDEa protein overexpressed in cultured cells and nuclear redistribution upon apoptosis induction. However, the final proof that CIDE binds to the homologous domain on DFF45 hence opposing its inhibitory effect on the DFF40 nuclease, which subsequently cleaves DNA, is still missing. Owing to its mitochondrial and nuclear localization, and interaction with mitochondrial UCP1, CIDEA may serve as a sensor of energy production in mitochondria and after the signal, overcome caspases to induce apoptosis independently of caspases cleavage. However, it seems that this is not the case.
Our findings of CIDEa redistribution during incumbent apoptosis, induced either by CIDEa overexpression or in synergy with other apoptotic initiators, make the model, in which the DFF45/DFF40 complex enters the nucleus and CIDEa has to migrate therein to initiate its dissociation, more plausible.

Exact pathway leading to CIDEa redistribution is unclear; however, we perceive the different mode of action for each stimulus as the culprit for more pronounced redistribution of CIDEa after valinomycin treatment. Main target of camptothecin, DNA- topoisomerase I complex, is clearly located in the nucleus. Result of the interaction, i.e. DNA strand breaks, could lead to release of mitochondrial proteins, including CIDEa, only via indirect means and after nuclear signaling to mitochondria. On the other hand, valinomycin is a potassium uniporter that causes collapse of mitochondrial membrane potential and its whole transformation into ΔpH *(Dlasková et al. 2008b)*, hence it likely triggers release of mitochondrial proteins as a result of mitochondrial network transformation.

6. CONCLUSIONS

1. Cancer cells exhibits low respiratory flux in the presence of glucose. Glucose deprivation resulted in elevated respiration. In this thesis, I proposed that tree distinct mechanisms regulate mitochondrial respiration of cancer cells: *i)* glucose inhibition, *ii)* substrate flux at the level of TCA cycle, *iii)* level of mitochondrial mass.

2. Crabtree effect exists in breast cancer cells. In this study it was clearly shown, that glucose presence possess the inhibitory effect on the mitochondrial respiration. Therefore, Crabtree effect can be interpreted as an active inhibition of respiration by glucose at the level of mitochondrial respiratory enzymes.

3. Survival of oxygen limitation of breast cancer cells is dependent on glucose presence. Further, lack of glucose inhibits the downregulation of mitochondrial biogenesis under the hypoxia.

4. Individual UCP isoform mRNA levels varied by up to four orders of magnitude in rat and mouse tissues.

5. UCP2 mRNA content was relatively high (0.4 to 0.8 pg per 10 ng of total mRNA) in rat spleen, rat and mouse lung, and rat heart.

6. Levels of the same order of magnitude were found for UCP3 mRNA in rat and mouse skeletal muscle, for UCP4 and UCP5 mRNA in mouse brain, and for UCP2 and UCP5 mRNA in mouse white adipose tissue.

7. Significant differences in pattern were found for rat vs. mouse tissues, such as the dominance of UCP3/UCP5 vs. UCP2 transcript in mouse heart and vice versa in rat heart; or UCP2 (UCP5) dominance in rat brain contrary to 10-fold higher UCP4 and UCP5 dominance in mouse brain.

8. We have confirmed mitochondrial localization of CIDEa and the role of CIDE-C domain in mitochondrial localization.

9. We observed redistribution, enhanced upon treatment with camptothecin or valinomycin, of CIDEa to nucleus.

10. We hypothesize that CIDEa is sequestered in mitochondria while transfer of this protein from mitochondria into nucleus intensifies or even initiates apoptosis.

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8. LIST OF PUBLICATIONS

Publications related to the thesis:

Mitochondrial bioenergetic adaptations of breast cancer cells to aglycemia and hypoxia.
Smolková, K., Bellance, N., Scandurra, F., Genot, E. Gnaiger, E., Plecitá-Hlavatá, L., Ježek, P., Rossignol, R. *Int J Biochem Cell Biol.* 2009, 41, revised version submitted, 2009.

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Publications not directly related to the thesis:

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