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SUMMARY OF DOCTORAL THESIS

Effect of selected nutrients on skeletal muscle mitochondrial metabolism

Účinek vybraných živin na mitochondriální metabolismus kosterního svalu

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CONTENTS

LIST OF ABBREVIATIONS

AMPK	adenosine monophosphate-activated protein kinase
ATP	adenosine triphosphate
CoA	coenzyme A
DAG	diacylglycerol
DNA	deoxyribonucleic acid
ETC	electron transport chain
FA	fatty acids
$FADH_2$	reduced form of flavin adenine dinucleotide
FFA	free fatty acids
H_2O_2	hydrogen peroxide
IMCL	intramyocellular lipids
IMTG	intramyocellular triacylglycerols
IR	insulin resistance
mtDNA	mitochondrial DNA
NADH	reduced form of nicotinamide adenine dinucleotide
PA	palmitic acid
PPAR	peroxisome proliferator-activated receptors
PUFA	polyunsaturated free fatty acids
RNA	ribonucleic acid
ROS	reactive oxygen species
SRC	spare respiratory capacity
T2D	type 2 diabetes
TAG	triacylglycerols
TCA	tricarboxylic acid
TPP	triphenylphosphonium
UCP	uncoupling protein

SUMMARY

Skeletal muscle plays an important role in the maintenance of whole-body metabolic homeostasis. Metabolic alterations of skeletal muscle contribute to the pathogenesis of a wide range of human diseases, such as obesity, type 2 diabetes and hypertension. Relative excess and suboptimal composition of nutrients negatively affect skeletal muscle metabolism and a better understanding of mechanisms involved in these changes is of central importance. The aim of the work presented in this thesis was to explore cell viability and mitochondrial respiratory parameters following experimentally induced changes in the availability or composition of selected nutrients (fatty acids and glutamine). We attempted to elucidate the mechanisms responsible for the observed changes, such as mitochondrial DNA (mtDNA) damage, or nuclear receptors activation. The studies were performed *in vitro* on skeletal muscle cell culture models. In addition, we examined mitochondrial function and fat accumulation in skeletal muscle of vegans, i.e. subjects consuming a strict plant-based diet.

Using C2C12 skeletal muscle cells we studied the effects of free fatty acids (FFA). We found that relatively low doses of saturated palmitic acid increased hydrogen peroxide production and induced mtDNA damage, mitochondrial respiratory dysfunction and cell death in myoblasts. Differentiated myotubes were more resistant to this lipotoxic effect and despite observed mtDNA damage mitochondrial respiration and cell viability were not compromised. Mitochondria-targeted antioxidants MitoQ and MitoTEMPOL did not prevent palmitic acid-induced damage. In the same model we also showed that unsaturated FFA effectively protect cells against the lipotoxic action of palmitic acid but this effect is not mediated by an activation of peroxisome proliferator-activated receptors δ (PPARδ). In addition to FFA, we also studied the effect of different doses of the amino acid glutamine in primary human skeletal muscle cells. We found that levels consistent with moderate clinical hypoglutaminemia are optimal for the proliferation of myoblasts and efficient oxidative phosphorylation of both myoblasts and myotubes. High levels of glutamine then uncoupled mitochondrial respiration.

In addition, we showed that metabolic benefits of a diet strictly avoiding animal products, particularly higher insulin sensitivity, are not associated with changes in mitochondrial density or fat accumulation in skeletal muscle.

We believe that our results contribute to the understanding of the effects of nutrients, particularly saturated and unsaturated fatty acids and glutamine, on skeletal muscle energy metabolism. A better understanding of the cellular biology and pathophysiology associated with changes in the availability of these nutrients can provide a framework for evidence-based prevention and treatment of many pathological states.

SOUHRN

Kosterní sval hraje významnou roli v udržování metabolické homeostázy celého organismu. Metabolické změny v kosterním svalu přispívají k patogenezi celé řady onemocnění, jako je obezita, diabetes 2. typu a hypertenze. Nadbytek a nevhodné složení živin negativně ovlivňují metabolismus kosterního svalu. Porozumění mechanismům, kterými k tomu dochází, je tedy důležitým cílem metabolického výzkumu. Cílem této práce bylo sledovat viabilitu buněk kosterního svalu a parametry mitochondriální respirace po experimentálně indukovaných změnách v dostupnosti nebo složení vybraných živin (mastných kyselin a glutaminu). Pokusili jsme se také objasnit mechanismy zodpovědné za pozorované změny, jako je poškození mitochondriální DNA (mtDNA) nebo aktivace jaderných receptorů. Jako *in vitro* model kosterního svalu byly použity kultivované svalové buňky. Dále jsme sledovali mitochondriální funkci a akumulaci tuku v kosterním svalu u veganů, tedy jedinců konzumujících striktně rostlinnou stravu.

Na svalových buňkách linie C2C12 jsme studovali účinky volných mastných kyselin (VMK). Zjistili jsme, že již relativně nízké dávky nasycené kyseliny palmitové zvýšily produkci peroxidu vodíku, indukovaly poškození mtDNA a mitochondriální respirační dysfunkci a snížily viabilitu myoblastů. Diferencované myotuby byly více rezistentní vůči tomuto lipotoxickému účinku a navzdory signifikantnímu poškození mtDNA nedošlo k poškození mitochondriální respirace ani snížení viability. Mitochondriálně cílené antioxidanty MitoQ a MitoTEMPOL nebyly schopné zabránit poškození vyvolanému kyselinou palmitovou. Na stejném modelu jsme ukázali, že nenasycené VMK efektivně chrání buňky před lipotoxickým účinkem kyseliny palmitové, nicméně tento účinek není zprostředkován aktivací jaderných receptorů peroxisome proliferator-activated receptors δ (PPARδ). Dále jsme studovali účinky různých koncentrací aminokyseliny glutaminu na lidských svalových buňkách. Zjistili jsme, že koncentrace glutaminu odpovídající mírné hypoglutaminémii je optimální pro proliferaci myoblastů a pro účinnou oxidativní fosforylaci myoblastů i myotub. Vyšší hladiny glutaminu již vedly k odpřažení mitochondriální respirace.

V poslední studii prezentované v této práci jsme ukázali, že jedinci konzumující striktně rostlinnou stravu mají vyšší inzulínovou sensitivitu než omnivorní kontroly, nicméně množství mitochondrií a tuku v kosterním svalu těchto jedinců se od kontrol neliší.

Věříme, že tyto výsledky přispějí k porozumění účinků živin, zejména nasycených a nenasycených mastných kyselin a glutaminu, na energetický metabolismus kosterního svalu. Lepší pochopení procesů probíhajících na buněčné úrovni, asociovaných se změnami dostupnosti těchto živin, může sloužit jako základ pro prevenci a léčbu mnoha patologických stavů.

1 INTRODUCTION

1.1 Skeletal muscle energy metabolism

Skeletal muscle comprises about 40% of total body mass in non-obese subjects and accounts for 20-30% of the resting metabolic rate [1]. Its metabolism largely contributes to energy and metabolic homeostasis of the whole organism.

Energy in skeletal muscle is derived mostly from glucose and fatty acids (FA). The main anaerobic pathway, occurring in the cytosol, is glycolysis. Aerobic metabolism (aerobic pathway for catabolism of glucose, catabolism of FA and amino acids) takes place in mitochondria and accounts for the majority of energy production.

1.1.1 Mitochondrial metabolism

Skeletal muscle is richly endowed with mitochondria and heavily reliant on oxidative metabolism for energy production [2]. Mitochondria generate most of the adenosine triphosphate (ATP) by a joint endeavour of reactions of ß-oxidation of FA and the tricarboxylic acid cycle (TCA) occurring in the mitochondrial matrix, and oxidative phosphorylation, which takes place in the inner mitochondrial membrane [3].

Oxidative phosphorylation

The oxidation of substrates generates reducing equivalents NADH and $FADH_2$, each containing a pair of high-energy electrons. The electrons are transferred from NADH or $FADH_2$ to molecular oxygen through a series of electron carriers located in the mitochondrial inner membrane, called the electron transfer chain (ETC). Molecular oxygen is the final acceptor of electrons and is reduced to water. The energy that is released as the electrons flow down the ETC is used to pump protons out across the mitochondrial inner membrane. The resulting distribution of protons generates a substantial transmembrane electrical potential and a smaller pH gradient that together create a protonmotive force, whose energy is used by the ATP synthase to make ATP [3].

Protons can also return to the matrix through pathways independent of ATP synthase (proton leak pathways), such as through nonspecific proton leak or via uncoupling proteins (UCP) [4]. In both cases, mitochondrial respiration is uncoupled and redox energy is wasted as heat rather than being used to synthetize ATP [5].

Mitochondria as a source of reactive oxygen species

The mitochondrial ETC is the major site of reactive oxygen species (ROS) production within the cell. Superoxide is produced continually as a byproduct of normal respiration by the one-electron reduction of molecular oxygen [6]. The superoxide is rapidly converted into hydrogen peroxide (H_2O_2) by compartment-specific superoxide dismutases. In the presence of ferrous or cuprous ions H_2O_2 can form the highly reactive hydroxyl radical which damages all classes of biomolecules [7].

Cells possess effective antioxidant defence mechanisms to prevent ROS-mediated damage, however, when mitochondrial ROS production exceeds the capacity of these mechanisms, ROS accumulate and damage cellular proteins, lipids, and nucleic acids. This state is defined as oxidative stress [8]. Mitochondria are not only a source of ROS but are also becoming targets to ROS-mediated damage [6]. Mitochondrial DNA (mtDNA) is especially sensitive to this damage because its proximity to the site of ROS formation, the lack of histones and limited ability to repair damaged DNA compared to nuclear DNA [9, 10]. Apart from their role in cellular damage, ROS also play an important role in redox signaling, i.e. in the maintenance of normal cellular functions [8, 11].

Excessive mitochondrial ROS production is thought to underlie a variety of pathologies associated with neurological degenerative diseases, obesity, diabetes, cardiovascular diseases or aging [4].

1.1.2 Metabolism of nutrients in skeletal muscle

Skeletal muscle plays an important role in the systemic regulation and metabolism of nutrients. It has a critical role in glycemic control, it is the main

site of FA utilization and provides the largest reserve of protein. The primary sources of energy for skeletal muscle are glucose and FA.

1.1.2.1 Fatty acids

The major types of FA in the circulation and in the tissues of mammals are long chain (14-18 carbon atoms) and very-long-chain (20-26 carbon atoms) FA with varying degrees of saturation [12]. Dietary fats typically comprise 30-40% of energy intake and consist mostly of long-chain FA esterified in triacylglycerols (TAG) [13]. For convenience, the term fatty acids will be used to designate "long-chain fatty acids", unless indicated otherwise.

FA are the main metabolic fuels for skeletal muscle. In skeletal muscle they are stored in the form of TAG packaged into cytoplasmic lipid droplets and referred to as intramyocellular TAG (IMTG) or intramyocellular lipids (IMCL). Mitochondria constitute the main subcellular compartments where FA degradation occurs. The lipid droplets are located in the close proximity to the mitochondria [14].

FA are supplied to skeletal muscle in the form of free FA bound to albumin, or derived from TAG in chylomicrons or very-low-density lipoproteins. The uptake of FA from the circulation occurs via both passive diffusion and protein-mediated transport, the latter supposed to account for the majority of FA uptake [13]. Once inside the cell, metabolism of FA starts by their activation via conversion to fatty acyl-coenzyme A (CoA) by the activity of the acyl-CoA synthetase. Depending on energy demand, these acyl-CoA either enter mitochondria for oxidation or are re-esterified and stored in TAG. The flux through β-oxidation in skeletal muscle appears to be controlled largely at the level of entry of acyl groups into mitochondria, i.e. at the level of enzyme carnitine palmitoyltransferase 1 (CPT1) [15].

1.1.2.2 Glutamine

Glutamine is the most abundant amino acid in human plasma (600-700 μM) and in the intracellular pool of free amino acid in skeletal muscle (~20 mM) [16]. Skeletal muscle produces most of the endogenous glutamine in the body [17]. Glutamine has many essential metabolic functions, such as precursor of

urinary ammonia, in the maintenance of acid-base status and in inter-organ nitrogen transfer for the biosynthesis of nucleotides, amino sugars and glutathione [18]. In tissue cultures, glutamine is an essential component of media for proliferating cells [19, 20].

Low muscle and plasma glutamine concentrations are observed in patients with sepsis, trauma and after major surgery [21, 22]. Although glutamine is a nonessential amino acid, it was suggested that it may become a conditionally essential during critical illness [16], where the requirements for glutamine may exceed its endogenous production. Therefore it was assumed that glutamine supplementation may offer therapeutic benefits in catabolic states and this effect was indeed demonstrated in many studies (reviewed in [16, 23]). However, several recent studies have not fully supported this assumption [24, 25].

1.2 Skeletal muscle metabolic dysfunction induced by fatty acids

Excess of FA and their inadequate composition are considered important factors responsible for metabolic dysfunction in skeletal muscle and a large part of work presented in this thesis is focused on mechanisms of FA action in skeletal muscle cells. Therefore this chapter mainly summarizes the current knowledge about the effects of different types of FA on skeletal muscle metabolism and mechanisms of their action. To distinguish between esterified and non-esterified FA in the biological systems, the latter are commonly called free FA (FFA).

Increased levels and/or fluxes of FFA occur in obesity [26]. FFA accumulate in tissues not designed for fat storage, a phenomenon described as ectopic fat deposition [27] and have various adverse effects in these tissues known as lipotoxicity [28]. The broadly studied and discussed pathological conditions associated with excess FFA are insulin resistance (IR) and impaired insulin-stimulated glucose disposal, the latter associated mainly with the skeletal muscle. These conditions are key components of type 2 diabetes (T2D) and the metabolic syndrome and considerable research effort has been made to determine the role of FFA in their development.

1.2.1 Mechanisms of free fatty acids action in skeletal muscle

Several mechanisms have been suggested to be involved in the development of FFA-induced IR and cellular dysfunction in skeletal muscle. More than 50 years ago Randle and colleagues proposed the concept of the glucose-fatty acid cycle [29]. Over the years many other mechanisms have been suggested, with central role of intracellular accumulation of lipid metabolites, mitochondrial oxidative capacity and oxidative stress.

1.2.1.1 Intracellular accumulation of lipid metabolites

A number of studies in both animals and humans reported that an accumulation of IMTG in skeletal muscle strongly correlates with IR [30, 31]; but this is true only in untrained individuals. Endurance-trained athletes are often extremely insulin sensitive despite a high content of IMTG and this observation was referred to as the athlete's paradox [32]. Moreover, it was shown that increasing IMTG does not always induce IR [33]. Therefore, it is now generally accepted that not IMTG accumulation itself but rather FFA-derived active lipid metabolites, such as diacylglycerol (DAG), ceramide or fatty acyl-CoA, are harmful for skeletal muscle. The association between accumulation of active lipid species (DAG and/or ceramide) and the inhibition of insulin action was demonstrated in the skeletal muscle from obese insulin-resistant individuals, healthy people after a lipid infusion and in the skeletal muscle of high fat-fed mice [34-36]. In vitro studies on skeletal muscle cells showed that specifically long-chain saturated FFA induced the synthesis of DAG and ceramide [37]. DAG and ceramide may also activate inflammatory pathways [38] and apoptosis [39].

The accumulation of FFA and their metabolites in skeletal muscle may result from the imbalance between FFA supply (cellular uptake), storage in TAG (lipolysis and lipid synthesis) and mitochondrial oxidation. Intact insulin sensitivity despite high IMTG in endurance-trained subjects has been explained by a higher turnover rate of the IMTG pool and a more efficient coupling of lipolysis to mitochondrial fat oxidation, which may reduce the accumulation of lipotoxic intermediates [40].

1.2.1.2 Mitochondrial oxidative capacity

Mitochondrial content and/or function were shown to be compromised in obese and T2D subjects [41-44] and in skeletal muscle of lean, insulin-resistant offspring of T2D subjects [45]. Based on these and other similar studies it has been assumed that impaired mitochondrial oxidative capacity plays a pivotal role in intracellular accumulation of FFA and their metabolites and the development of IR in skeletal muscle [46]. However, a number of studies in animals and humans is incompatible with this concept and observed lipid-induced IR in skeletal muscle without an impairment of mitochondrial function [47-49] or with impairment which developed long time after the establishment of IR [50]. These studies argue against the concept that muscle lipid accumulation and IR are mediated by a deficiency in mitochondrial oxidative capacity.

In fact, a few years ago, an alternative hypothesis connecting fatty acid oxidation to lipid-induced IR in skeletal muscle has been proposed, declaring excessive rather than reduced β-oxidation [51]. This model proposed that lipid oversupply into the mitochondria drives an increase in mitochondrial ß-oxidation that exceeds the capacity of the TCA cycle and the ETC, leading to an incomplete fatty acid oxidation and intramitochondrial accumulation of byproducts of oxidation, mitochondrial stress, IR and cellular dysfunction [51]. Other animal studies also revealed an increased rather than decreased mitochondrial biogenesis and mitochondrial oxidative capacity in high fat-fed rodents [52].

1.2.1.3 Mitochondrial (oxidative) stress

A few years ago, production of ROS has emerged as an important link between excess FFA, mitochondria and IR. Studies in high fat-fed rodents and obese people showed increased mitochondrial ROS production in skeletal muscle in association with IR and without signs of mitochondrial respiratory deficiency [50, 53, 54]. These studies suggest that an increased mitochondrial ROS production and altered cellular redox state are major determining factors in the loss of insulin sensitivity associated with high fat intake or obesity.

Mitochondrial dysfunction is then considered to be a consequence of altered cellular metabolism and IR [50]. This is in agreement with the above-mentioned theory of mitochondrial lipid overload with elevated β-oxidation, as an increased oxidation of FFA can lead to mitochondrial stress, increased ROS production and cellular damage.

In studies on cultured muscle cells, excessive ROS and oxidative damage of mtDNA were proposed as initial events leading to mitochondrial/cellular dysfunction, IR and apoptosis in myotubes [55, 56]. These effects were observed for saturated FFA and were abolished by targeting DNA repair enzymes into mitochondria [55] or by overexpressing catalase [56].

Based on current evidence it is clear that interaction of FFA with mitochondria plays an important role in cellular events induced by lipid overload in skeletal muscle. However, it is difficult to conclude whether increasing fatty acid oxidation in mitochondria would be beneficial for muscle metabolism and insulin sensitivity or not. Muoio and colleagues proposed that increasing the flux through β-oxidation could be beneficial only in parallel with increased energy expenditure which reduces the pressure on mitochondrial ETC and prevents excessive ROS production [57]. In general, increased energy expenditure is an effective mechanism to maintain insulin sensitivity and other cellular functions in skeletal muscle exposed to lipid overload and exercise is a simple way to achieving it. On animal model, it was shown that increased energy expenditure by activation of adaptive thermogenesis in muscle was associated with resistance to obesogenic effect of high fat diet [58].

1.2.1.4 Specific effects of unsaturated free fatty acids

Studies into the mechanisms of FFA action in cultured muscle cells revealed that their effects are dependent on the type of FFA. Saturated palmitic acid induced IR, inflammation, mitochondrial damage, oxidative stress and apoptosis in skeletal muscle cells [55, 59] whereas unsaturated FFA did not cause these changes and even showed protective effects against saturated FFA-induced damage.

It has been proposed that unsaturated FFA can protect cells against lipotoxicity by promoting FFA incorporation into TAG [60, 61] and/or oxidation in mitochondria [59, 62]; thereby decreasing their availability for metabolic conversions to active lipid metabolites. However, exact mechanisms of this action need to be elucidated.

1.2.1.5 Peroxisome proliferator-activated receptors

FFA can regulate energy metabolism in skeletal muscle cells through binding to peroxisome proliferator-activated receptors (PPAR). These nuclear receptors act as transcription factors and control the expression of genes involved in glucose and lipid metabolism. Unsaturated FFA and their metabolites have been reported as effective natural ligands and activators of these receptors [63]. Three isoforms of PPAR with tissue-specific expressions and functions were identified: PPARα, β/δ and γ.

PPARδ is the most abundant isoform in skeletal muscle [64] and preferential/increased fat oxidation is an important metabolic effect of PPARδ activation [65]. Ablation of PPARδ in skeletal muscle of mice led to obesity and diabetes [66]. An activation of PPARδ with a synthetic agonist protected mice against high-fat diet-induced IR in skeletal muscle [67] and prevented FFA-induced inflammation and IR in muscle cells [68]. Therefore, PPARδ has gained attention as a potential target for treatment of metabolic abnormalities in skeletal muscle associated with fat accumulation.

On the other hand, oral administration of the PPARδ agonist to rodents worsened insulin-stimulated glucose transport in skeletal muscle [69]. Moreover, PPARδ-mediated increase in muscle mitochondrial oxidative capacity was observed in high fat-fed mice together with the establishment of IR [52]. Cell culture studies are then inconsistent regarding the involvement of PPARδ in the protective effects of unsaturated FFA against lipotoxicity [59, 70]. Therefore, the role of PPARδ activation under the conditions of lipid overload, whether by unsaturated FFA or synthetic agonist, needs further study.

2 AIMS

The general aim of this thesis was to contribute to the understanding of the effects of selected nutrients on skeletal muscle energy metabolism, particularly mitochondrial respiration. Most of the work was focused on the effects of different FFA, minor part was concentrated on the effect of glutamine. C2C12 mouse myoblast cell line and primary human skeletal muscle cells were used as *in vitro* models of skeletal muscle. Population with strictly defined nutritional habits (vegans) was also studied.

Specific aims:

- To elucidate the effects of saturated FFA on the viability and mitochondrial respiration in C2C12 myoblasts and myotubes.

- To test the ability of mitochondria-targeted antioxidants to prevent saturated FFA-induced damage in C2C12 myoblasts.

- To determine whether PPARδ activation is involved in the protective effects of unsaturated FFA against saturated FFA-induced damage in C2C12 myotubes.

- To assess whether metabolic benefits observed in a population consuming strict plant-based diet (vegans) are associated with changes in mitochondrial density and fat accumulation in skeletal muscle.

- To elucidate the effects of hypoglutaminemia on the rate of proliferation and on mitochondrial respiration in primary human myoblasts and myotubes.

3 RESULTS AND DISCUSSION

3.1 List of original publications

1. Palmitate-induced cell death and mitochondrial respiratory dysfunction in myoblasts are not prevented by mitochondria-targeted antioxidants

Jana Patková, Michal Anděl and Jan Trnka

Cellular Physiology and Biochemistry 2014; 33(5): 1439-1451. IF 3.55

2. Protective effect of unsaturated fatty acids on palmitic acid-induced toxicity in skeletal muscle cells is not mediated by PPARδ activation

Jana Tůmová, Lucia Mališová, Michal Anděl and Jan Trnka

Lipids 2015; 50(10): 955-964. IF 1.85

3. Higher insulin sensitivity in vegans is not associated with higher mitochondrial density

Jan Gojda, Jana Patková, Martin Jaček, Jana Potočková, Jan Trnka, Pavel Kraml and Michal Anděl

European Journal of Clinical Nutrition 2013; 67(12): 1310-1315. IF 2.95

4. Normalizing glutamine concentration causes mitochondrial uncoupling in an in vitro model of human skeletal muscle

Adéla Krajčová, Jakub Žiak, Kateřina Jiroutková, Jana Patková, Moustafa Elkalaf, Valer Džupa, Jan Trnka and František Duška

Journal of Parenteral and Enteral Nutrition 2015; 39(2): 180-189. IF 3.15

3.2 Comments on the articles and discussion of results

3.2.1 Effects of different fatty acids on energy metabolism in C2C12 skeletal muscle cells

Excess of FFA and/or their inadequate composition are considered as significant triggers of obesity-related metabolic complications in skeletal muscle. However, mechanisms of their action on cellular level, especially of different types of FFA, are not fully understood. Therefore, the first two studies presented in this thesis are centred on examining the effects of FFA and mechanisms of their action in skeletal muscle cells, with focus on energy metabolism.

Saturated FFA were reported to cause cellular damage in skeletal muscle cells [55, 71] which can eventually lead to a cell death [55, 72]. Although various mechanisms may be involved in their lipotoxic action, changes in mitochondrial respiration associated with increased ROS production seem to play a central role. Therefore, the aim of **the first study** was to clarify the effect of saturated palmitic acid (PA) on the viability and mitochondrial respiration in C2C12 myoblasts and myotubes and to test the ability of mitochondria-targeted antioxidants MitoQ and MitoTEMPOL to prevent PA-induced damage.

Cells were exposed to 100 μM PA conjugated to BSA or control BSA for 18 h. MitoQ or MitoTEMPOL were added to myoblasts 1 h prior to the addition of PA. Viability, integrity of mtDNA, basic parameters of mitochondrial respiration and ROS production were assessed.

We found that PA induced similar mtDNA damage in myoblasts and myotubes, which was however associated with reduced mitochondrial respiration, increased hydrogen peroxide production (by about 50%) and a decreased cell viability (by about 35%) only in myoblasts. This effect of PA was not previously reported in undifferentiated myoblasts. Damage of mtDNA associated with increased oxidative stress, some features of mitochondrial dysfunction, and apoptosis, were reported in rat L6 myotubes, but with relatively higher doses of PA compared to our study [55, 73]. We used 100 μM concentration of PA, which we considered as a physiologically relevant dose

[74]. With this dose we observed signs of mtDNA damage in myotubes but without concomitant reduction of mitochondrial respiration or cell viability. We propose that this higher resistance of myotubes to lipotoxic effects of PA compared to myoblasts could be explained by their higher spare respiratory capacity (SRC), which was 75% of maximal respiration in myotubes compared to only 53% in myoblasts. SRC is critical for survival and function of cells, especially in conditions of acute or chronic stress [75, 76].

Previous studies reported protective effects of MitoQ and MitoTEMPOL against mitochondrial oxidative damage in various cell types [77-79]. In our study, similar doses of these mitochondria-targeted antioxidants did not prevent the cell death in PA-treated myoblasts, although MitoTEMPOL prevented mtDNA damage. Moreover, both antioxidants as well as control propyltriphenylphosphonium (propylTPP, a cation moiety of antioxidant molecules) markedly inhibited mitochondrial respiration. Acute toxicity of TPP-conjugated compounds as well as TPP moiety itself for mitochondrial respiration was recently reported in mouse kidney mesangial cells [80]. These findings indicate that TPP-conjugated antioxidants should be used carefully and their potential effects on cellular bioenergetics should be taken into account.

In summary, our results showed PA induced mtDNA damage in myoblasts and myotubes, which was however associated with reduced mitochondrial respiration and decreased cell viability only in myoblasts. Mitochondria-targeted antioxidants were unable to prevent PA-induced cell death in myoblasts.

Compared to saturated FFA, unsaturated FFA do not cause mitochondrial and cellular damage and even protect skeletal muscle cells against deleterious effects of saturated FFA [59, 62, 81]. FFA are endogenous ligands of PPAR, transcription factors regulating the expression of genes involved in lipid and energy metabolism [82]. However, the role of FFA as natural ligands of PPAR and regulation of PPAR in conditions of lipid overload are poorly understood. The objective of **the second study** was to determine whether activation of PPARδ, the most common PPAR subtype in skeletal muscle, is involved in mediating protective effects of unsaturated FFA on saturated FFA-induced

damage in C2C12 myotubes. Another aim was to assess the impact of different FFA and PPARδ activation on mitochondrial respiration.

Cells were treated for 24 h with saturated PA, mono- and polyunsaturated FFA (oleic acid, linoleic acid and α-linolenic acid) and their combinations. Total FFA concentration was 100, 300, 600 or 1000 μM. PPARδ agonist GW501516 or antagonist GSK0660 were added to some of the treatments. Changes in mRNA expressions of known PPARδ-target genes were used as a marker of PPARδ activation. Viability and mitochondrial respiration were also assessed.

Both mono- and polyunsaturated FFA prevented PA-induced cell death in our experiments. We confirmed that unsaturated FFA are effective activators of PPARδ in C2C12 myotubes as they increased mRNA expressions of PPARδ-target genes to the same degree as PPARδ selective agonist GW501516. Saturated PA was a weak activator of PPARδ as was previously suggested [63]. In contrary to our expectations, when unsaturated FFA were mixed with PA, their effect on PPARδ activation was blocked, i.e. it remained at the levels observed for PA alone. We hypothesize that this effect could be a result of an interaction between PA and unsaturated FFA, since in combination of PA with GW501516, PPARδ activity was markedly increased. These findings indicate that PPARδ activation is not involved in the protective effect of unsaturated FFA in C2C12 myotubes. Other mechanisms, independent of PPARδ, should explain beneficial effects of unsaturated FFA. Indeed, a recent study showed that oleic acid prevented PA-induced cellular damage through an adenosine monophosphate-activated protein kinase (AMPK)-dependent mechanism, without an involvement of PPAR [70]. The protective effect of unsaturated FFA may also involve the channeling of PA into TAG. This mechanism was reported in cultured muscle cells for oleic acid [59, 72].

Unsaturated FFA at moderate physiological concentrations (300 μM), but not PA, mildly uncoupled mitochondrial respiration. This effect might be, at least in part, responsible for their beneficial effects as mild uncoupling increases energy expenditure and can therefore increase fatty acids removal and decrease their availability for metabolic conversions and other pathways leading to cellular damage [83]. Similar uncoupling effect was observed also for agonist GW501516.

In conclusion, we found that PPARδ activation is not involved in the protective effects of unsaturated FFA against PA-induced lipotoxicity in C2C12 myotubes.

3.2.2 Underlying mechanisms of metabolic benefits of strictly plant-based diet

Development of IR and overall metabolic dysfunction is linked with higher intake of fat but also with inadequate composition of fat in the diet. Saturated fat, a source of which are mainly animal products, appears to have the most significant detrimental impact on insulin sensitivity [84]. People consuming a plant-based diet (vegetarians and vegans) were shown to have more favourable metabolic profile, i.e. lower fasting glucose, plasma lipid profile, higher insulin sensitivity [85-87], and also a lower IMCL content [86], compared with omnivores. However, the causes of these metabolic differences are not clear. The aim of **the third study** was to investigate whether metabolic benefits, particularly insulin sensitivity, observed in vegans are associated with changes in IMCL content and altered mitochondrial density in skeletal muscle. Composition of plasma FFA pool was also assessed.

Eleven vegans and ten matched omnivorous controls were enrolled in a case–control study. The composition of plasma FFA pool and insulin sensitivity were assessed, and skeletal muscle biopsies were performed (vastus lateralis). IMCL content and mitochondrial density markers (activity of citrate synthase and relative amount of mtDNA) were measured in muscle samples.

We showed that vegans had lower fasting plasma glucose and insulin levels and higher insulin-stimulated glucose disposal, which is in agreement with previous studies [85, 86]. IMCL content did not significantly differ between groups although there was a trend toward a lower IMCL content in vegans. However, it is now widely discussed that a high turnover rate of IMTG and more efficient coupling of lipolysis to fatty acid oxidation may play a more important role in preserving insulin sensitivity than content of IMCL *per se* [40, 88]. Markers of mitochondrial content did not significantly differ in muscle samples from vegans and controls. These findings are in agreement with a novel

hypothesis that lipid accumulation and IR in skeletal muscle are not triggered by a deficiency in mitochondrial oxidative capacity as was previously suggested [46, 89].

Vegans were reported to have a higher intake of polyunsaturated fat, and lower intake of saturated fat [86]. We assessed plasma levels of FFA and vegans had indeed higher levels of plasma polyunsaturated FFA (PUFA), although the levels of plasma saturated FFA did not differ between groups. Increased dietary intake and plasma levels of PUFA may be responsible for beneficial effects of the plant-based diet as suggested by some groups [86, 90] but this association deserves further investigation.

In conclusion, we found that metabolic benefits observed in vegans are not associated with alterations in IMCL content or changes in mitochondrial density in skeletal muscle.

3.2.3 Effects of hypoglutaminemia on energy metabolism in human skeletal muscle cells

Glutamine has been the focus of scientific interest because of its unique physiological role in humans, animals and cultured cells. It has been used clinically as a nutrition supplement in a wide range of wasting diseases [18]. In cell cultures, glutamine is required for cell growth [19, 20] and proliferating cells use glutamine as both nitrogen donor and energy substrate [91, 92]. It could be hypothesized that hypoglutaminemia impairs proliferation of cells and their energy metabolism. In **the fourth study** we studied the effects of different glutamine concentrations on the rate of proliferation and on mitochondrial respiration in human skeletal muscle cells; both myoblasts and myotubes were examined.

Human myoblasts were isolated from skeletal muscle biopsy samples and exposed for 20 days to 6 different glutamine concentrations, resembling various degrees of clinical hypoglutaminemia (0, 100, 200, and 300 μM), a normal glutamine concentration in human plasma (500 μM), and a high concentration, similar to that used in cell cultures (5000 μM). Half of these cells were allowed to differentiate into myotubes and energy metabolism was assessed in both

myoblasts and myotubes. The proliferation rate of myoblasts was determined by manual counting of cells every 5 days.

This study showed a dependence of human myoblasts proliferation rate on glutamine concentration. Compared to previously mentioned studies [19, 20], in our study myoblasts grown in glutamine-free media remained viable and did proliferate. However, significant limitation of the proliferation rate was observed at glutamine concentrations below 200 μM, while the fastest proliferation rate was observed for 300 μM. This is interesting since this dose is close to the concentrations observed in patients with protracted critical illness [93] and seems to be optimal for myoblasts proliferation. Further increase, up to 5000 μM glutamine, did not bring any additional benefit in terms of myoblast proliferation.

The most interesting finding from mitochondrial respiration analysis in myoblasts and myotubes is the uncoupling effect of high doses of glutamine. Highly coupled respiration was observed in both myoblasts and myotubes cultured in the presence of 200-300 μM glutamine, while the highest concentrations of glutamine decreased the efficiency to ~60-75% in both myoblasts and myotubes. It is known that an increased mitochondrial uncoupling leads to increased energy expenditure. In line with this, glutamine supplementation has been shown to increase fat oxidation in critically ill patients [94] and to increase energy expenditure and fat oxidation in healthy subjects [95]. However, mitochondrial uncoupling may also be a result of an uncontrolled leak resulting from glutamine-induced mitochondrial damage, as possible mitochondrial toxicity of glutamine was previously reported [96]. If the mitochondrial uncoupling is due to increased nonspecific proton leak or controlled leak through UCP is not clear from our data and needs to be examined in future studies.

In conclusion, we showed that glutamine concentrations consistent with moderate clinical hypoglutaminemia represent optimal condition for myoblast proliferation and for efficiency of aerobic phosphorylation in an in vitro model of human skeletal muscle.

4 CONCLUSIONS

We showed that saturated PA is more cytotoxic for undifferentiated skeletal muscle cells (myoblasts) then for differentiated myotubes. PA caused mtDNA damage in both cell types, which was associated with reduced mitochondrial respiration, increased hydrogen peroxide production and cell death only in myoblasts. Despite the fact that oxidative stress seems to play a role in the lipotoxic effect of PA, mitochondria-targeted antioxidants MitoQ and MitoTEMPOL were unable to prevent PA-induced cell death in myoblasts. Moreover, they markedly inhibited mitochondrial respiration. This finding indicates that they interfere with cellular bioenergetics and in some cases their application may be more harmful than beneficial. We also showed that unsaturated FFA effectively protect cells against the cytotoxic effect of PA and that this effect is not mediated by the activation of PPARδ. In studies concerning the effects of glutamine we found that levels of glutamine consistent with moderate clinical hypoglutaminemia are optimal for the proliferation of human myoblasts as well as for the efficiency of oxidative phosphorylation of both myoblasts and myotubes. Increasing glutamine concentrations above that level caused mitochondrial uncoupling.

In addition, we showed that metabolic benefits of a vegan diet, such as lower fasting glucose or higher insulin sensitivity, are not associated with changes in fat accumulation or mitochondrial density in skeletal muscle. These findings are in agreement with a hypothesis that lipid accumulation and IR in skeletal muscle are not mediated by a deficiency in mitochondrial oxidative capacity [50, 57].

To summarize the contributions of this thesis, we believe that our findings provide some new insights into the effects of different FFA and glutamine on energy metabolism in skeletal muscle cells and into mechanisms of their action. Our results and data from other studies indicate that saturated FFA (especially PA) when standing alone are very toxic, however, in combination with unsaturated FFA their toxicity is prevented. Mitochondrial respiration then seems to play a central role in the effects of both types of FFA and also glutamine.

5 ANNEXES

Articles not included in the thesis with IF:

1. Evaluation of lipofuscin-like pigments as an index of lead-induced oxidative damage in the brain

Jana Patková, Max Vojtíšek, Jan Tůma, František Vožeh, Jana Knotková, Pavlína Šantorová and Jiří Wilhelm

Experimental and Toxicologic Pathology 2012; 64(1-2): 51-56. IF 2.62

2. Transplantation of Embryonic Cerebellar Grafts Improves Gait Parameters in Ataxic Lurcher Mice

Václav Babuška, Zbyněk Houdek, Jan Tůma, Zdeňka Purkartová, Jana Tůmová, Milena Králíčková, František Vožeh and Jan Cendelín

Cerebellum 2015; 14(6): 632-641. IF 2.72

3. Excess of free fatty acids as a cause of metabolic dysfunction in skeletal muscle

Jana Tůmová, Michal Anděl and Jan Trnka

Physiological Research 2015 (in press). Review. IF 1.29

Articles not included in the thesis without IF:

Účinek nasycených a nenasycených volných mastných kyselin na inzulínovou rezistenci a metabolismus kosterního svalu

Jana Patková, Jan Trnka and Michal Anděl

Diabetologie, metabolismus, endokrinologie, výživa 2012; 15(2): 131-137. Review.

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