**Charles University in Prague**
**Third Faculty of Medicine**
Department of Nutrition

**Doctoral thesis**

# Effect of selected nutrients on skeletal muscle mitochondrial metabolism

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## DECLARATION

Hereby I declare that this thesis is based on experiments performed at the Department of Nutrition of the Third Faculty of Medicine, Charles University in Prague, in the Laboratory for Metabolism and Bioenergetics, during my Ph. D. studies and was written by me. All sources of information are reported in the list of references. This thesis was not used to obtain any other or similar degree.

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# CONTENTS

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AA | amino acids |
| ACC | acetyl-CoA carboxylase |
| ADP | adenosine diphosphate |
| AMP | adenosine monophosphate |
| AMPK | adenosine monophosphate-activated protein kinase |
| ATP | adenosine triphosphate |
| $Ca^{2+}$ | calcium ion |
| $CO_2$ | carbon dioxide |
| CoA | coenzyme A |
| CoASH | coenzyme A not attached to an acyl group |
| CPT | carnitine palmitoyltransferase |
| DAG | diacylglycerol |
| DHA | docosahexaenoic acid |
| DNA | deoxyribonucleic acid |
| $e^-$ | electron |
| EPA | eicosapentaenoic acid |
| ETC | electron transport chain |
| FA | fatty acids |
| FABPc | cytosolic fatty acid–binding protein |
| FAD | oxidized form of flavin adenine dinucleotide |
| $FADH_2$ | reduced form of flavin adenine dinucleotide |
| FAT | fatty acid translocase |
| FATP | fatty acid transport protein |
| FFA | free fatty acids |
| GLUT | glucose transporters |
| $H^+$ | hydrogen ion |
| $H_2O$ | water |
| $H_2O_2$ | hydrogen peroxide |
| IKK-ß | IκB kinase-ß |
| IMCL | intramyocellular lipids |
| IMTG | intramyocellular triacylglycerols |
| IR | insulin resistance |
| IRS-1 | insulin receptor substrate 1 |
| JNK | c-Jun N-terminal kinase |
| LPL | lipoprotein lipase |
| mtDNA | mitochondrial DNA |
| $NAD^+$ | oxidized form of nicotinamide adenine dinucleotide |
| NADH | reduced form of nicotinamide adenine dinucleotide |

| | |
|---|---|
| NF-κB | nuclear factor kappa B |
| $O_2$ | dioxygen |
| Δp | protonmotive force |
| PA | palmitic acid |
| PDH | pyruvate dehydrogenase |
| Pi | inorganic phosphate |
| PI3K | phosphoinositide 3-kinase |
| PKB | protein kinase B |
| PKC | protein kinase C |
| 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 well tolerated and 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 selected nutrients (i.e. 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 lipotoxickému účinku kyseliny palmitové 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 buňkami dobře tolerovaná a 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, přestože množství mitochondrií a tuku v kosterním svalu těchto jedinců se od kontrol neliší.

Věříme, že tyto výsledky přispějí k porozumění účinků vybraných živin (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 therefore largely contributes to energy and metabolic homeostasis of the whole organism.

A striking characteristic of skeletal muscle energy metabolism is the capacity to rapidly modulate the rate of energy production and substrate utilization in response to changing energy demands. It must increase energy turnover hundredfold when shifting from the resting state to the fully-activated state within a few milliseconds. In other situations, skeletal muscle has to be able to maintain a moderately increased energy turnover during prolonged periods [2]. To cope with these diverse tasks, skeletal muscle is composed of muscle cells with different metabolic and contractile properties, i.e. different fiber types [2]. They range from slow, highly oxidative fibers, rich in mitochondria, which are optimized for sustained movements to fast, glycolytic fibers, with less mitochondria, suited for short explosive movements (reviewed in [3]).

Energy in skeletal muscle is derived mostly from glucose and fatty acids (FA); additional substrates are amino acids. The chemical energy trapped within the bonds of substrate molecules is extracted as adenosine triphosphate (ATP), which is produced in anaerobic and aerobic metabolic pathways. The main anaerobic pathway, occurring in the cytosol, is glycolysis. In this reaction glucose is broken down into pyruvate, which is then converted into lactate. This process does not require oxygen but does not yield much energy. In the presence of oxygen pyruvate is transported into mitochondria and converted to acetyl-coenzyme A (CoA) and can be used in aerobic metabolism.

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

#### 1.1.1.1 Mitochondria as producers of energy

Skeletal muscle is richly endowed with mitochondria and heavily reliant on oxidative metabolism for energy production [4]. Mitochondria generate most of the 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. The TCA (also known as the Krebs cycle) is the common final pathway for all energy-providing macromolecules (Fig. 1) [5].

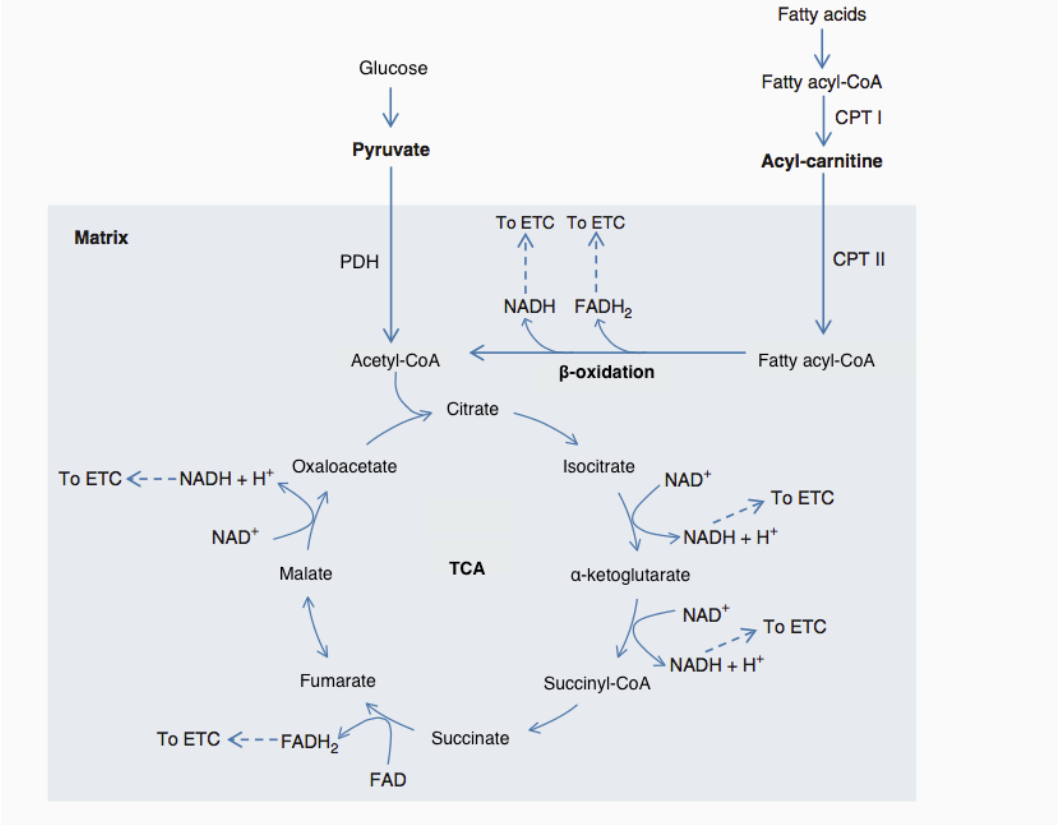


**Figure 1:** Oxidative pathways for substrates degradation in the matrix of mitochondria with a central role of tricarboxylic acid cycle (TCA). Main substrates used to provide energy in skeletal muscle (glucose and fatty acids) are shown. Amino acids can also serve as energy substrates, they are metabolized to acetyl-CoA or intermediates of the TCA cycle. For explanation of abbreviations, see List of abbreviations on page 3. Adapted from Salabei et al. 2014 [6].

*Oxidative phosphorylation*

The oxidation of substrates generates reducing equivalents NADH and $FADH_2$ (Fig. 1), 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 (Fig. 2). 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 (Δp), whose energy is used by the ATP synthase to make ATP. Thus, the oxidation of substrates and the phosphorylation of ADP are coupled by a proton gradient across the mitochondrial inner membrane. The gradient is also used to drive other processes than ATP synthesis, such as import of proteins, adenine nucleotides, $Ca^{2+}$ and other molecules into mitochondria [7]. The ETC is primarily composed of four protein complexes numbered I to IV and two mobile molecules (coenzyme Q and cytochrome c) carrying electrons between the main complexes (Fig. 2) [5].

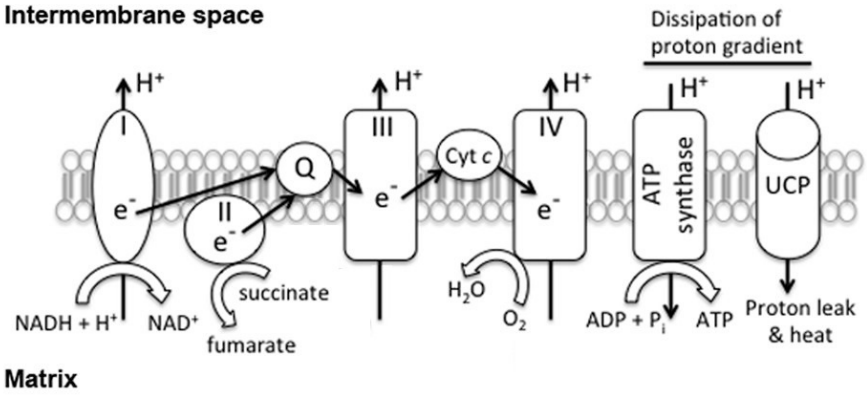


**Figure 2:** A schematic diagram of the mitochondrial electron transport chain (complexes I to IV) and the pathways dissipating the proton gradient (ATP synthase and uncoupling protein, UCP). Q, coenzyme Q; Cyt c, cytochrome c. For explanation of all abbreviations, see List of abbreviations on page 3. Adapted from Ho et al. 2012 [8].

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). These proteins allow passive proton transport through the membrane and can be induced and activated by various stimuli [9]. In both cases, mitochondrial respiration is uncoupled and redox energy is wasted as heat rather than being used to synthetize ATP [10]. Proton leak has a significant influence on energy metabolism of the whole organism. It has been estimated that 10 - 25% of the resting metabolic rate is attributed to respiration that supports mitochondrial proton leak [11].

The efficiency with which reducing equivalents are converted to ATP by oxidative phosphorylation is known as the coupling efficiency. It varies with ATP demand or type of substrate and also depends on the cell type, but it is usually fairly high, up to 90%, in skeletal muscle cells [12].

#### 1.1.1.2 Mitochondria as a source of reactive oxygen species

In addition to their crucial role in ATP generation, mitochondria also take part in other important cellular functions, such as calcium homeostasis, apoptosis or the generation of reactive oxygen species (ROS). In the next part, I will focus on reviewing their role in the generation of ROS and maintenance of cellular redox environment, as it is relevant to the experimental work presented in this thesis.

The mitochondrial ETC is the major site of ROS production within the cell. Superoxide is produced continually as a byproduct of normal respiration by the one-electron reduction of molecular oxygen [13]. Respiratory complex I and to a lesser extent complex III have been identified as the major sources of superoxide in mitochondria [14]. The superoxide is released either into the mitochondrial matrix or into the cytosol and is rapidly converted into hydrogen peroxide ($H_2O_2$) by compartment-specific superoxide dismutases. $H_2O_2$ can easily diffuse throughout the cell and can oxidize susceptible protein thiols inside and outside mitochondria. In the presence of ferrous or cuprous ions $H_2O_2$ can form the highly reactive hydroxyl radical which damages all classes of biomolecules

and initiates lipid peroxidation cascades in membranes [7]. Many other radical and non-radical species can be formed by reactions of these ROS with other molecules in biological systems, such as a damaging oxidant peroxynitrite formed in reaction of superoxide with nitric oxide [10].

*Oxidative stress*

Cells possess effective antioxidant defence mechanisms to prevent ROS-mediated damage, consisting of a large family of enzymes and low-molecular-weight compounds [15]. 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 [16]. Mitochondria are not only a source of ROS but are also becoming targets to ROS-mediated damage [13]. 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 [17,18]. A chronic excessive ROS production leads to a vicious cycle of mitochondrial functional decline, further ROS generation, and extensive cellular injury.

*Redox signaling*

Although ROS indisputably contribute to mitochondrial and cellular damage, they also play an important role in redox signaling, in the maintenance of normal cellular functions [16, 19]. According to current understanding, the mechanism of redox signaling involves the $H_2O_2$-mediated reversible oxidation of cysteine residues on proteins which alters the activity of enzymes and transcription factors [20]. Redox signaling is required for numerous cellular processes, such as regulation of cellular growth and differentiation, regulation of inflammation or cellular response to hypoxia [16, 21]. Although quite little is currently known about mitochondrial redox signaling, it has been suggested that mitochondrial $H_2O_2$ efflux could act as a retrograde signal to the cell, which reports on mitochondrial $\Delta p$ or the redox state of NADH pool, and thus enables integrating mitochondrial function with that of the rest of the cell [13].

It appears that both the type of ROS and its local concentration determine whether redox signaling or oxidative stress-induced damage occurs [16]. Excessive mitochondrial ROS production is thought to underlie a variety of pathologies associated with neurological degenerative diseases, obesity, diabetes, cardiovascular diseases or aging [9].

*Regulation of ROS production*

Mitochondrial ROS production is favoured by high levels of reduction of the respiratory electron carriers and by a large electrochemical potential gradient (high $\Delta p$) [10]. Mild uncoupling and therefore a slight reduction of $\Delta p$ might significantly decrease superoxide production from ETC [14]. Indeed, artificial uncouplers such as 2,4-dinitrophenol, which dissipate the gradient across membrane, were shown to decrease the rate of superoxide production in mitochondria [22]. UCP3, a member of UCP family highly expressed in skeletal muscle, has been shown to respond to overproduction of matrix superoxide by catalyzing mild uncoupling which decreases ROS production from ETC at the cost of slightly lowered efficiency of oxidative phosphorylation [14]. UCP3 catalyzes proton conductance of the mitochondrial inner membrane in the presence of specific activators, such as ROS or their byproducts and perhaps FA [23]. Therefore, UCP3 in skeletal muscle has been suggested to play a role in the regulation of ROS production and protection against oxidative damage rather than in adaptive energy uncoupling, such as in the case of UCP1 [24].

### 1.1.2 Metabolism of nutrients in skeletal muscle

Skeletal muscle plays an important role in the systemic regulation and metabolism of nutrients, mostly because of its large total mass. 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. Amino acids derived from muscle protein degradation serve as additional substrates, however, in well-fed subjects their contribution to aerobic metabolism is low [25].

#### 1.1.2.1 Glucose

Glucose is a fundamental energy source for all eukaryotic cells and it is an important fuel for contracting skeletal muscle, especially during high intensity exercise [26]. Skeletal muscle stores glucose in the form of glycogen and is the largest glycogen depot in the body. It accounts for about 80% of postprandial insulin-stimulated glucose disposal [27, 28] and thus regulates the blood glucose levels.

Glucose passes through the plasma membrane and enters skeletal muscle cells by facilitated diffusion via glucose transporters (GLUT). Several isoforms of GLUT were identified in skeletal muscle. GLUT1 mediate the basal glucose uptake into the muscle fibers. They reside primarily in plasma membrane and were shown to be present in large amounts in vascular endothelia, among other cells [29]. The principal glucose transporters, highly expressed in skeletal muscle, are GLUT4, which mediate both basal and stimulated glucose uptake [30, 31]. In the basal state, most of the GLUT4 is located to intracellular vesicular storage compartments, whereas upon stimulation, GLUT4 are translocated to the plasma membrane and T-tubules and mediate glucose entry into the cell [32]. The stimuli can be hormonal (insulin) or mechanical (muscle contraction), but also of other type (i.e. hypoxia or metabolic stress) [33]. The effects of insulin and contraction on glucose uptake are additive. Their signaling pathways have several convergence points, which are intensively studied, especially because of benefits of exercise on skeletal muscle metabolism, when insulin action is impaired [26].

Several more members of the GLUT family were found to be expressed in skeletal muscle, i.e. GLUT12, which was suggested to share some of the features of GLUT4 [29] or GLUT5, which is actually fructose, not glucose, transporter [29, 34]. However, relatively little is known about the importance and regulation of these GLUT isoforms in skeletal muscle.

Once glucose has been transported across the plasma membrane, it is rapidly phosphorylated to glucose 6-phosphate in a reaction catalyzed by hexokinase II. This is the first step in the metabolism of glucose via either pathways responsible for energy generation or conversion to glycogen. Under physiological conditions, approximately two-thirds of all glucose 6-phosphate is converted to glycogen, and

one-third enters glycolysis [35]. Pyruvate, the end-product of glycolysis, enters the mitochondria via mitochondrial pyruvate carriers [36]. The mitochondrial multienzyme complex pyruvate dehydrogenase (PDH) then converts pyruvate to acetyl-CoA that can enter the TCA cycle.

The transport of glucose across the plasma membrane in skeletal muscle is the initial and, under many physiological conditions, the rate-limiting event in glucose metabolism [30]. Given the critical role of skeletal muscle in blood glucose control, a disturbance of glucose transport into muscle has a large impact on whole-body glucose homeostasis. Glucose uptake is compromised whenever the response of skeletal muscle to insulin action is impaired; this condition is called insulin resistance (IR) and it occurs in obesity or type 2 diabetes [37]. Causes of IR in skeletal muscle are multiple and involve an excess of FA, obesity-induced inflammation or ROS production [38-40].

#### 1.1.2.2 Fatty acids

*Structure and common types of fatty acids*

FA are carboxylic acids with an aliphatic chain. FA in biological systems usually contain an even number of carbon atoms, typically between 14 and 24. The alkyl chain may be saturated (saturated FA) or it may contain one (monounsaturated FA) or more (polyunsaturated FA) double bonds. The properties of FA and of derived lipids are markedly dependent on their chain length and the degree of saturation. Short chain length and unsaturation enhance the fluidity of FA and the structures which are built from them, such as biological membranes [5].

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 [41] (listed in Tables 1-3). The most common FA found in human plasma include saturated palmitic acid, monounsaturated oleic acid and polyunsaturated linoleic acid [42]. A similar pattern is observed also in the plasma of rodents [43]. However, the composition of plasma and tissue FA can significantly differ between individuals with respect to dietary fat intake [44]. Dietary fats typically comprise 30-40% of energy intake and consist mostly of

long-chain FA esterified in triacylglycerols (TAG) [45]. For convenience, the term fatty acids will be used in this thesis to designate "long-chain fatty acids", unless indicated otherwise.

**Table 1:** Saturated fatty acids.

| Formula | Systematic name | Trivial name |
|---|---|---|
| 12:0 | dodecanoic | lauric |
| 14:0 | tetradecanoic | myristic |
| **16:0** | **hexadecanoic** | **palmitic** |
| 18:0 | octadecanoic | stearic |

**Table 2:** Monounsaturated fatty acids.

| Formula | Systematic name | Trivial name |
|---|---|---|
| 16:1 (n-7) | hexadec-9-enoic | palmitoleic |
| **18:1 (n-9)** | **octadec-9-enoic** | **oleic** |
| 18:1 (n-7) | octadec-11-enoic | vaccenic |
| 20:1 (n-9) | eicos-11-enoic | gondoic |
| 22:1 (n-9) | docos-13-enoic | erucic |

**Table 3:** Polyunsaturated fatty acids.

| Formula | Systematic name | Trivial name |
|---|---|---|
| **18:2 (n-6)** | **octadeca-9,12-dienoic** | **linoleic (LA)** |
| 18:3 (n-3) | octadeca-9,12,15-trienoic | α-linolenic (ALA) |
| 20:3 (n-6) | eicosa-8,11,14-trienoic | dihomo-γ-linolenic (DGLA) |
| 20:4 (n-6) | eicosa-5,8,11,14-tetraenoic | arachidonic (AA) |
| 20:5 (n-3) | eicosa-5,8,11,14,17-pentaenoic | EPA |
| 22:6 (n-3) | docosa-4,7,10,13,16,19-hexaenoic | DHA |

**Tables 1 - 3:** The major types of fatty acids in the circulation and in the tissues of mammals. The three most common fatty acids found in plasma are in bold.

*Uptake and catabolism of FA*

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, so that FA can be readily hydrolysed and oxidised [46, 47].

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, from which are liberated by endothelial lipoprotein lipase (LPL). 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 [45]. Three main types of fatty acid binding/transport proteins have been identified, i.e. plasma membrane fatty acid binding protein (FABPpm), fatty acid translocase (FAT)/CD36 and fatty acid transport proteins (FATP), out of which FATP1 and FATP4 are the most abundant in skeletal muscle [48]. The content of these transporters in plasma membrane increases in response to acute mechanical stimuli (muscle contraction) [49] or hormonal stimuli (insulin) [50, 51]. It means that they facilitate uptake of FA to clear lipids from the circulation in postprandial state or to provide substrates when the metabolic demands of muscle are increased. This regulation seems to be similar to the regulation of glucose uptake by glucose transporters [45].

Once inside the cell, FA are bound by cytosolic fatty acid binding proteins (FABPc) and can undergo a number of metabolic fates [52]. FABPc are found at high concentrations (0.5 - 5% of cytosolic protein) and function as an intracellular buffer of FA [48]. Metabolism of FA starts by their activation via conversion to fatty acyl-coenzyme A (acyl-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. FA stored in TAG can undergo lipolysis on stimuli and become available substrates for oxidation.

The first step in FA oxidation is the transport of acyl groups into mitochondria. Acyl-CoA, which cannot cross the inner mitochondrial membrane,

is converted to acyl-carnitine by muscle isoform of carnitine palmitoyltransferase 1 (CPT1) on the outer mitochondrial membrane. The acyl-carnitine is then translocated across the inner mitochondrial membrane by carnitine:acylcarnitine translocase (CACT) and in the matrix converted back to acyl-CoA by CPT2. FA are then catabolized via β-oxidation by sequential removal of two-carbon units from the acyl chain. The end product of each cycle of β-oxidation is acetyl-CoA, which can enter the TCA cycle. β-oxidation provides ATP not just via acetyl-CoA production, but also by direct generation of reducing equivalents which supply electrons to ETC [53].

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 CPT1 [54]. CPT1 activity is inhibited by malonyl-CoA, which is created from acetyl-CoA by acetyl-CoA carboxylase (ACC) [55]. ACC2 is the major isoform expressed in skeletal muscle [56]. The regulation of ACC2 activity is complex; it is activated e.g. by insulin or cytosolic citrate and inhibited by AMP-activated protein kinase (AMPK) [56, 57]. β-oxidation flux is also controlled by the redox state of reducing equivalents and by acetyl-CoA/CoASH ratio [53].

The β-oxidation pathway accomplishes the complete degradation of saturated FA with even number of carbon atoms. The oxidation of unsaturated FA requires the participation of additional enzymes (an isomerase and a reductase). Oxidation of FA occurs also in the peroxisomes. A primary function of peroxisomal β-oxidation is to shorten very-long-chain FA to facilitate their degradation in the mitochondria. Peroxisomes are also capable of performing α-oxidation, e.g. of branched chain FA [58].

The majority of FA taken up by muscle is either oxidized in mitochondria or stored as TAG. Part of FA is incorporated into phospholipids and serves as building blocks of biological membranes [45]. Apart from these functions, FA can be metabolized to lipid second messengers, such as prostaglandins, ceramides or diacylglycerols, which are important signaling molecules [59]. FA are also involved in regulation of cellular processes via protein acylation (palmitoylation and myristoylation) [60, 61] or via regulation of gene transcription [52].

Because of the myriad of functions, chronic imbalance in FA fluxes and/or disturbed FA metabolism in skeletal muscle are associated with a variety of metabolic abnormalities and pathologies, with IR standing currently at the forefront of metabolic research interest.

#### 1.1.2.3 Metabolic flexibility

As was mentioned above, skeletal muscle uses FA and glucose as the main metabolic fuels, but FA predominate during fasting state, while glucose dominates in the fed (insulin-stimulated) state. The ability of skeletal muscle to dynamically change between FA and glucose as energy sources, and to adjust fuel oxidation to fuel availability, is defined as metabolic flexibility [62].

Competition between glucose and FA for their oxidation in muscle was first described by Randle and colleagues [63] who showed that the utilization of one nutrient inhibits the use of the other. This mechanism was called the glucose-fatty acid cycle or the Randle cycle. In this study and many other studies conducted since this first report, it was revealed that the competition between glucose and FA occurs at several levels in the cell, largely at the level of phosphofructokinase (PFK, regulation of glycolytic flux), PDH (regulation of glucose oxidation) and CPT1/malonyl-CoA (regulation of FA oxidation) [63, 64]. The competition may also occur within the matrix of mitochondria in interactions between β-oxidation and TCA cycle and by delivering $FADH_2$ and NADH to different loci within the ETC [65]. Modulation of expression of key enzymes in the pathways of glucose and FA metabolism has been recently also shown to play a role [64].

Loss of capacity to switch easily between glucose and FA oxidation is termed metabolic inflexibility and was observed in skeletal muscle in association with obesity, IR and type 2 diabetes in humans [62, 66] and after high-fat diet in rats [43] (Fig. 3).

Interestingly, it was shown that cultured skeletal muscle cells established from healthy subjects retain metabolic characteristics of the donor in terms of metabolic flexibility [67].

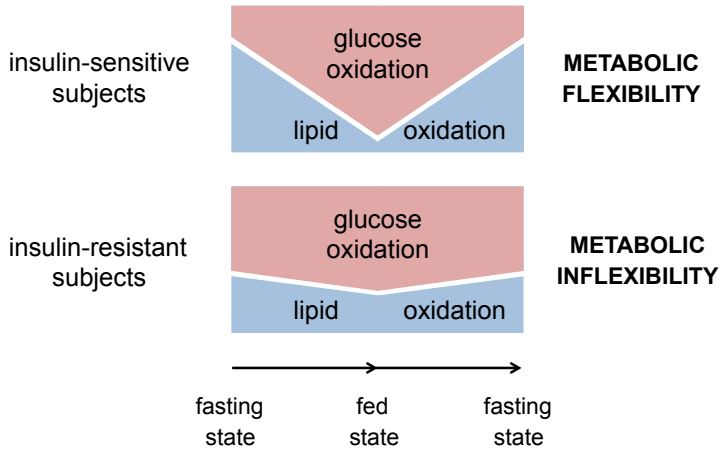


**Figure 3:** A concept of metabolic inflexibility in skeletal muscle. In healthy subjects transition from the fasting to the fed state is accompanied by decreasing the rate of lipid oxidation and increasing the rate of glucose oxidation. This substrate switch is impaired in obese, insulin-resistant subjects who lack the ability to switch to glucose oxidation in the fed (insulin-stimulated) state. Based on Eckardt et al. 2011 [68].

#### 1.1.2.4 Amino acids

Amino acids (AA) occur in nature in the form of proteins or as free AA. The vast majority of AA in the human body is present in the form of proteins, of which 40-45% is located in skeletal muscle, primarily as contractile proteins. Less than 2% of total AA in the body exist in their free forms and approximately half of the free AA pool is present in skeletal muscle. There is a continuous exchange of AA between the free pool and protein compartments, called protein turnover [69]. Skeletal muscle actively participates in the handling and metabolism of AA and exchanges them with other tissues [70].

AA are not primarily metabolic fuels but they can be converted to TCA cycle intermediates or their precursors and metabolized to $CO_2$ and $H_2O$. Only six AA are metabolized in skeletal muscle. These are the branched-chain AA (BCAA) leucine, isoleucine and valine, and glutamate, aspartate and asparagine [71]. After period of starvation (AA from net breakdown of muscle proteins) or following ingestion of protein-containing meal (dietary AA) AA are metabolized in muscle and used largely for *de novo* synthesis of glutamine and alanine, which are then released into the circulation. They represent safe nitrogen carriers responsible for

the elimination of amino groups from muscle and serve as precursors for gluconeogenesis [70]. Other AA are also released from skeletal muscle during catabolic breakdown, but glutamine and alanine account for about one-half to two-thirds of released amino acid nitrogen [72]. Only leucine and part of isoleucine molecule can be directly oxidized to provide energy in muscle as they are converted to acetyl-CoA, other AA are converted to TCA cycle intermediates.

AA metabolism in skeletal muscle can be affected by multiple factors, such as dietary intake of proteins and AA, physical (in)activity and acute or chronic illness.

*Glutamine*

Glutamine is the most abundant AA in human plasma (600-700 μM) and in the intracellular pool of free AA in skeletal muscle (~20 mM) [72]. Skeletal muscle produces most of the endogenous glutamine in the body [73]. 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 [74]. In addition, glutamine serves as the preferred oxidative fuel for rapidly proliferating cells, such as enterocytes and lymphocytes [75, 76]. Glutamine has been reported to have also cell- and immune- modulating capacity [77].

Low muscle and plasma glutamine concentrations are observed in patients with sepsis, trauma and after major surgery [78, 79]. Although glutamine is a nonessential AA, it was suggested that it may become a conditionally essential during critical illness [72,80], 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 [72, 81]). However, several recent studies have not fully supported this assumption [82, 83].

In tissue cultures, glutamine is an essential component of media for proliferating cells [84, 85, 86].

## 1.2 Skeletal muscle metabolic dysfunction induced by fatty acids

Excess of FA and/or their inadequate composition are considered extremely 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 and doses 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).

### 1.2.1 Obesity-induced changes in the flux of free fatty acids

Systemic changes in FFA fluxes occur in obesity, a state characterized by excessive fat accumulation. Although the aetiology of obesity is multifactorial, the main risk factors involve an increase in total calorie intake, consumption of high-sugar and high-fat diet, and a sedentary lifestyle [87]. Obesity is frequently associated with metabolic dysfunction in various tissues, including liver, heart or skeletal muscle. Current evidence suggests that FFA are a significant factor contributing to this dysfunction [38, 88].

The adipose tissue plays an important role in buffering the flux of dietary fat into the circulation in the postprandial period by suppressing the release of FFA into the circulation and by increasing plasma TAG/FFA clearance. This buffering action limits an abnormal increase in plasma lipids and protects other tissues from the exposure to excessive lipid fluxes [89]. However, obesity is often associated with a dysfunctional adipose tissue. Obese subjects have been reported to have both an increased FFA release from adipose tissue and a reduction in postprandial plasma TAG/FFA clearance, with concomitant higher fasting and postprandial plasma FFA levels compared to lean subjects [90-92]. Increased levels and/or fluxes of FFA lead to their accumulation in tissues not designed for fat storage, a phenomenon described as ectopic fat deposition [93].

Dysfunctional adipose tissue due to obesity releases also other molecules negatively affecting skeletal muscle metabolism, such as adipokines or inflammatory molecules [39, 94].

#### 1.2.1.1 Systemic effects of free fatty acids excess

Excessive amounts of FFA accumulated in non-adipose tissues have various adverse effects known as lipotoxicity [95]. The broadly studied and discussed pathological conditions associated with excess FFA are IR and impaired insulin-stimulated glucose disposal, the latter associated mainly with the skeletal muscle. These conditions are key components of type 2 diabetes and the metabolic syndrome and considerable research effort has been made to determine the role of FFA in their development.

To investigate the effects of high FFA levels in humans or animal models several experimental approaches have been used: lipid-heparin infusion, which predominately releases unsaturated FFA from exogenous TAG; prolonged fasting, which is accompanied by an increased release of FFA from endogenous lipid stores and a long-term consumption of high-fat diet. All approaches have some limitations, but the dietary intervention best reflects the physiological situation in obese individuals. Time course studies to determine gradual changes in metabolism caused by diet are especially valuable. However, due to difficulties with control of all aspects of dietary intervention, an intravenous lipid infusion is often the preferred approach.

Experiments using lipid infusions and prolonged fasting demonstrated that increasing plasma FFA levels in lean individuals to the levels seen in obese ones induced lipid accumulation in skeletal muscle and IR [96, 97]. Dietary intervention studies with a consumption of high-fat diet also showed reduced insulin sensitivity and lipid accumulation in healthy volunteers [98] and rodents [99]. These findings indicate a close link between increased FFA levels/fluxes, ectopic lipid accumulation and IR.

Not only the amount but also the quality of dietary lipids is important in determining their effects in the body [100]. Different types of FFA, defined by the degree of saturation and the length of carbon chain, may induce different

metabolic responses [101]. Intervention studies in humans indicated that saturated fat significantly worsens insulin sensitivity, whereas monounsaturated and polyunsaturated fats have a less pronounced effect or even a potential to improve insulin sensitivity [102-104]. In line, animal models also demonstrated proinflammatory and insulin-antagonizing effects of saturated FFA [105, 106]. However, according to the KANWU study, the composition of fat is a factor in the induction of IR only when the daily energy intake from fat does not exceed a threshold of 35-40% of calorie intake. Above this threshold insulin sensitivity is influenced solely by the total amount of fat irrespective of its composition [100, 102].

These findings indicate that the composition of fat in lipid intervention studies should be considered as an important variable when investigating the metabolic impact of FFA.

#### 1.2.1.2 Type 2 diabetes

Type 2 diabetes (T2D) is an increasing world health problem and the parallel rapid growth of obesity and T2D underlines the link between these two conditions [107]. T2D is a chronic metabolic disorder defined by elevated blood glucose (hyperglycemia). Increased circulating FFA are also common in T2D [108, 109], nevertheless, it is not clear whether disordered lipid metabolism with elevated plasma FFA levels is a consequence of the diabetic state or plays a causal role in its development [110]. T2D is characterized by two features: 1) insulin resistance, defined as an impaired ability of insulin to suppress hepatic glucose output and to promote peripheral glucose disposal (predominantly in skeletal muscle) and 2) impaired function of pancreatic ß-cells such that insulin secretion is insufficient to match the degree of insulin resistance [111]. Although the cause of T2D is still unknown, it is clear that IR in skeletal muscle and liver plays a primary role in its pathogenesis before the failure of pancreatic ß-cells [35, 112]. As elevated plasma FFA were shown to induce IR both in liver and skeletal muscle [38] they can be considered as an important risk factor for the development of the syndrome. However, T2D is a multifactorial disease and a combination of genetic and many environmental risk factors may contribute to its pathogenesis [113].

### 1.2.2 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. Over the years many other mechanisms have been suggested, with central role of intracellular accumulation of lipid metabolites, mitochondrial oxidative capacity and oxidative stress.

#### 1.2.2.1 Randle cycle: competition between fatty acids and glucose

Randle and colleagues were the first to propose a connection between elevated FFA concentrations and reduction in glucose disposal in muscle [63]. Their study showed that an elevation in FFA supply to the diaphragm and isolated heart led to the glucose-fatty acid cycle (see chapter 1.1.2.3), resulting in an increased rate of fatty acid oxidation and inhibition of glucose utilization. They proposed a mechanism explaining the reduced glucose uptake by a model, where an increased oxidation of muscle fatty acids produces increased levels of intracellular acetyl-CoA and citrate, which then inhibit the activities of enzymes involved in glucose utilization, pyruvate dehydrogenase and phosphofructokinase. The lowering of pyruvate oxidation and glycolysis would then result in glucose-6-phosphate accumulation and inhibition of hexokinase II activity, resulting in increased intracellular glucose content and reduction in glucose uptake [114].

Subsequent studies have not fully confirmed Randle's hypothesis and have indicated that other mechanisms are likely involved in the FFA-induced impairment of glucose disposal in skeletal muscle, such as defects in insulin signaling pathways with concomitant inhibition of GLUT4 translocation [64, 115].

Although the role of impaired metabolic flexibility in skeletal muscle IR has been a subject of an intense research for years (reviewed in [116]), no clear conclusion seems to come from these studies.

#### 1.2.2.2 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 [117, 118]; 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 [119]. Moreover, it was shown that increasing IMTG does not always induce IR [120, 121] and can even increase insulin sensitivity [122]. 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 [99, 123,124]. *In vitro* studies on skeletal muscle cells confirmed this association [125-127]. Specifically long-chain saturated FFA were shown to induce the synthesis of DAG and ceramide, with the most potent inducer being palmitic acid [126].

DAG and ceramide are active signaling molecules. Increased levels of these metabolites are involved in the activation of both conventional and novel protein kinases C (PKC) isoforms and c-Jun N-terminal kinases (JNK), which in turn impair insulin-stimulated signaling cascades leading to impaired glucose uptake. Furthermore, through IκB kinase-ß (IKK-ß) and nuclear factor (NF)-κB they may activate inflammatory pathways with a concomitant production of proinflammatory cytokines, such as interleukin 6 (Fig. 4) [68, 128]. Ceramides are known initiators of the apoptotic cascade [129] and apoptosis induced by ceramide accumulation was reported in myotubes exposed to palmitic acid [130].

Although recent animal and human studies strongly support the key role of DAG and the activation of PKCθ in the pathogenesis of lipid-induced muscle IR [131, 132], there are also indications that an accumulation of DAG is not always associated with IR [120, 123]. Therefore, the role of this lipid species in muscle metabolism should be further examined, with emphasis on specific molecular species of DAG and their subcellular localization [123].

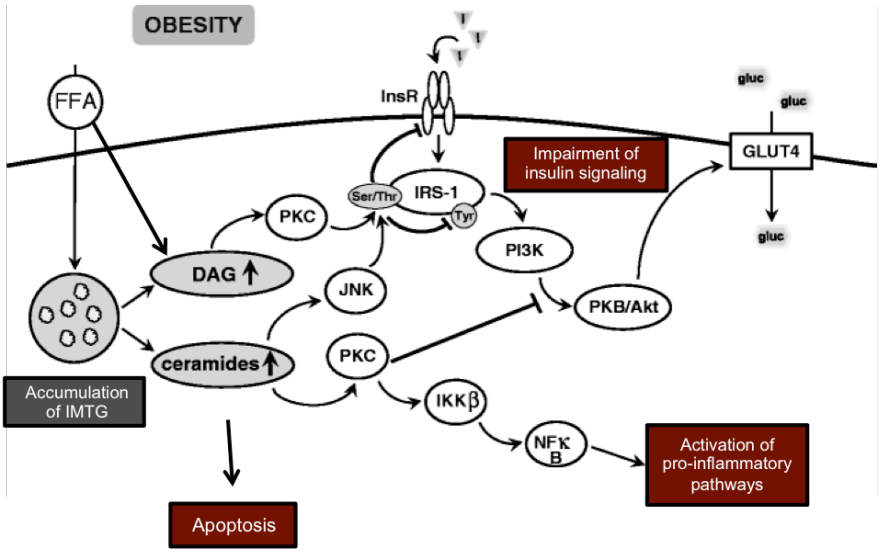


**Figure 4:** Intracellular signaling cascades leading to IR, inflammation and apoptosis induced by lipid metabolites DAG and ceramide. InsR, insulin receptor. For explanation of all abbreviations, see List of abbreviations on page 3. Adapted from Eckardt et al. 2011 [68].

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 [133]. Studies in obese humans and high fat-fed rats indeed revealed an enhanced transport of FFA into skeletal muscle associated with an increased IMTG content [134, 135] and a reduction in IMTG content after inhibition of FAT/CD36 in myotubes obtained from obese patients [136]. It was also shown that acute exercise or upregulation of TAG synthesis prevented lipid-induced impairments of insulin action in skeletal muscle and decreased DAG and ceramide accumulation [137, 138]. The role of mitochondrial fatty acid oxidation is a controversial topic and will be discussed in the next part.

#### 1.2.2.3 Mitochondrial oxidative capacity

Many different mitochondrial abnormalities have been reported in skeletal muscle of insulin-resistant obese and T2D subjects such as a deficiency of mitochondrial ETC [139], decreased expression of genes involved in oxidative metabolism and mitochondrial biogenesis [140, 141], decreased fat oxidation [142] or less abundant mitochondria with altered morphology [4, 143]. *In vivo* nuclear magnetic resonance studies revealed defects in mitochondrial oxidative phosphorylation with an accumulation of lipids in the skeletal muscle of lean, insulin-resistant offspring of T2D patients [144] or elderly, insulin-resistant individuals [145]. In addition, feeding a high-fat diet to healthy young men or mice was reported to decrease muscle mRNA levels of genes involved in the oxidative phosphorylation [146]. Based on these 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 [114, 147]. Evidence from cell culture and animal studies, which showed that increased oxidation of FFA in mitochondria, achieved by pharmaceutical or genetic approaches, reduced IMTG and lipid intermediates content and ameliorated IR in skeletal muscle in face of lipid overload [131, 148, 149] was in agreement with this hypothesis.

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 [150-152] or with impairment which developed long time after the establishment of IR [153]. In animal models, reduced oxidative phosphorylation due to a genetic modification of components of the ETC protected against the development of high-fat diet-induced IR and even increased insulin sensitivity in skeletal muscle [154, 155]. These findings argue against the concept that muscle lipid accumulation and IR are mediated by a deficiency in mitochondrial oxidative capacity. Another argument is based on the fact that skeletal muscle has a large respiratory reserve (spare capacity) to substantially increase substrate flux and ATP synthesis to meet a potential increase in energy demand. Most of the time, mitochondrial respiration in skeletal muscle is operating very far from its maximal capacity. Therefore it is

questionable if moderately decreased mitochondrial content or enzyme activities can influence the rate of fat oxidation and lipid accumulation when energy requirements are relatively low [156].

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 [43]. 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 and impaired insulin action and cellular dysfunction. Experimental evidence confirmed that reduced fatty acid uptake and catabolism in mitochondria prevented lipid-induced IR in myotubes and skeletal muscle of high fat-fed mice [43]. Other studies documented elevated incomplete fat oxidation associated with impaired insulin signaling in cultured myocytes from obese subjects and myocytes from lean subjects exposed to excess FFA [157]. Another animal studies also revealed an increased rather than decreased mitochondrial biogenesis and mitochondrial oxidative capacity in high fat-fed rodents [158, 159], pointing to an adaptation of lipid oxidation during lipid overload.

Studies in cultured skeletal muscle cells demonstrated decreased generation of ATP [160], reduced oxidation of FFA and intracellular lipid accumulation [161] associated with IR after prolonged exposure to saturated FFA. However, acute exposure (1h) of skeletal muscle cells to palmitic acid on the contrary induced ß-oxidation [162]. In another study transcriptional activation of pathways that increase fatty acid oxidation prevented DAG accumulation, inflammatory processes and development of IR induced by saturated FFA [163].

#### 1.2.2.4 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 [153, 164, 165]. Moreover attenuating mitochondrial ROS production protected against high-fat diet-induced IR [164, 166]. 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 [153]. 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.

Increased ROS production by saturated FFA was demonstrated also in cultured muscle cells [167]. Excessive ROS and oxidative damage of mtDNA were proposed as initial events leading to mitochondrial/cellular dysfunction, IR and apoptosis in myotubes [168, 169]. These effects were observed for saturated FFA and were abolished by targeting DNA repair enzymes into mitochondria [168] or by overexpressing catalase [169]. ROS were also shown to activate stress kinases, such as JNK and IKK-β, which have been linked to IR [170, 171] and this pathway may be a potential link between ROS and IR.

Several mechanisms of how the catabolism of FFA promotes mitochondrial ROS production have been proposed, such as excessive generation of reducing equivalents in β-oxidation, generation of intermediates and byproducts of β-oxidation that can inhibit enzymes that detoxify ROS or direct inhibition of the ETC by these intermediates [172]. Exposure to excess FFA coupled with physical inactivity may lead to intensive mitochondrial ROS production. In such model, increased FFA availability increases flux through β-oxidation and provides a high supply of electrons to ETC, while a lack of physical activity and the consequent low ATP demand favour a high protonmotive force, hyperpolarisation of mitochondrial membrane potential, inhibition of ETC and low respiration rate, i.e. conditions, which promote mitochondrial ROS formation [173].

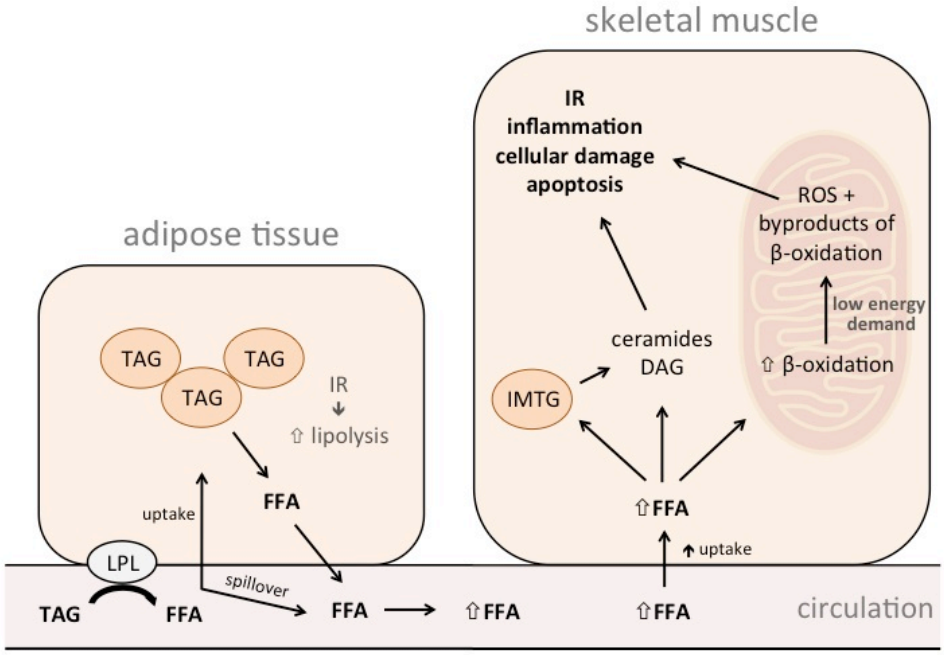


**Figure 5**: A schematic view of obesity-associated, FFA-induced IR and metabolic dysfunction in skeletal muscle. An increased release of FFA from dysfunctional adipose tissue and a reduction in postprandial plasma TAG/FFA clearance lead to higher circulating FFA levels/fluxes and higher uptake to skeletal muscle. FFA accumulated in skeletal muscle can be incorporated into IMTG or oxidized in mitochondria. Excess FFA can then be converted to active lipid metabolites, such as ceramide and DAG. Oversupply of FFA drives an adaptive increase in mitochondrial ß-oxidation, which, without an increase in energy demand, leads to incomplete fatty acid oxidation, increased redox pressure on the ETC and increased production of ROS, with ensuing oxidative stress and/or altered redox signaling, development of IR and cellular damage. For explanation of abbreviations, see List of abbreviations on page 3.

Based on this evidence it is clear that interaction of FFA with mitochondria plays an important role in cellular events induced by lipid overload in skeletal muscle. Even if impaired mitochondrial oxidative capacity is not the underlying mechanism of lipid accumulation and IR, there are indications that mitochondrial content, morphology and function are compromised by excess FFA in cell cultures, animal models and in obese subjects. These changes, however, may occur secondary to FFA-induced IR and alteration of cellular metabolism. ROS

are currently considered as early mediators responsible for FFA-induced IR and other metabolic abnormalities.

At this point 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 [156] which reduces the pressure on mitochondrial ETC and prevents excessive ROS production. 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. Regular exercise and physical activity are considered cornerstones in the prevention and treatment of numerous chronic conditions, including hypertension, obesity, T2D, and age-related muscle wasting [174]. 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 [175].

#### 1.2.2.5 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; as was observed in animal and human studies, where composition of fat in the diet was an important factor in the induction of IR (see chapter 1.2.1.1). Saturated palmitic acid induced IR, inflammation, mitochondrial damage, oxidative stress and apoptosis in skeletal muscle cells [128, 168, 176] whereas unsaturated FFA did not cause these changes and even showed protective effects against saturated FFA-induced damage. This protection has been observed mainly for monounsaturated oleic acid [128, 177] and to a certain degree also for polyunsaturated FFA [178].

It has been proposed that unsaturated FFA can protect cells against lipotoxicity by promoting FFA incorporation into TAG and thereby decreasing their availability for metabolic conversions to active lipid metabolites causing cellular damage and apoptosis [179]. Studies on muscle cells demonstrated that an excess of palmitic acid was poorly incorporated into TAG and caused IR and

apoptosis, in contrary to oleic acid, which was well incorporated into TAG and well tolerated. Moreover, oleic acid prevented deleterious action of palmitic acid by promoting its incorporation into TAG [125, 180]. In animal studies, directing FFA into TAG by polyunsaturated fat diet or increased synthesis of TAG in transgenic mice prevented FFA-induced IR in skeletal muscle [138, 181]. Both mono- and polyunsaturated FFA were shown to ameliorate saturated FFA-induced damage also by transcriptional activation of pathways that promote fat oxidation [128, 178, 180]. Altogether, different effects of saturated and unsaturated FFA can be explained by their different metabolic fates in the cell. Beneficial effects of unsaturated FFA seem to be mediated through an increased intracellular FFA disposal by promoting their storage in TAG and/or oxidation in mitochondria. The latter, however, should be associated with increased energy expenditure, as discussed above.

Unsaturated FFA were reported to improve insulin sensitivity in animals and humans also by increasing the unsaturation of skeletal muscle membrane phospholipids [182, 183]. Membrane lipid composition influences membrane fluidity and the function of integral membrane proteins and may therefore affect physiological mechanisms involved in FFA and glucose uptake. The fatty acid spectrum in skeletal muscle phospholipids and TAG was shown to reflect the composition of dietary fat [44, 184].

#### 1.2.2.6 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, while saturated FFA are only weak activators [185, 186].

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 [187]. Both PPARα and PPARδ share some target genes involved in fatty acid and glucose metabolism [188]; and preferential/increased fat oxidation is

an important metabolic effect of their activation. However, based on knock-out mice studies, PPARδ seems to play the dominant role in skeletal muscle [188, 189]. PPARδ is known to coordinate physiological adaptations of skeletal muscle in response to fasting and endurance exercise [190]. Ablation of PPARδ in skeletal muscle of mice led to obesity and diabetes [189]. An activation of PPARδ with a synthetic agonist protected mice against high-fat diet-induced IR in skeletal muscle [148] and prevented FFA-induced inflammation and IR in muscle cells [163]. Therefore, PPARδ has gained attention as a potential target for treatment of metabolic abnormalities in skeletal muscle associated with fat accumulation, mainly because of its favourable effects on fat oxidation and energy expenditure.

On the other hand, oral administration of the PPARδ agonist to rodents worsened insulin-stimulated glucose transport in skeletal muscle [191]. Moreover, PPARδ-mediated increase in muscle mitochondrial oxidative capacity was observed in high fat-fed mice together with the establishment of IR [159]. Cell culture studies are also inconsistent regarding the involvement of PPARδ in the protective effects of unsaturated FFA against lipotoxicity [128, 192]. Therefore, the role of PPARδ activation under the conditions of lipid overload, whether by unsaturated FFA or synthetic agonist, needs further study.

PPARγ is the least expressed isoform in skeletal muscle, however, its role in the maintenance of insulin sensitivity in skeletal muscle has been reported [193, 194] and should be further examined. An interesting question also is, how PPAR activity in skeletal muscle could be affected by composition of FFA in the diet or lipid intervention. However, it should be noted that the regulation of PPAR is complex and likely depends not only on the availability of their ligands but also on the presence of different co-regulators, phosphorylation status, etc. [195] and these factors can further complicate the elucidation of their role.

*Part of Introduction has been published in a review article (Tumova et al. 2015, see Annexes).*

# 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 Models used in the experiments

We undertook most of the studies on skeletal muscle cell culture models. Despite obvious shortcomings of cultured cells in terms of the lack of *in vivo* environment and interactions, they serve as a valuable experimental material to study the processes that occur at cellular and subcellular level. The extracellular environment can be precisely controlled *in vitro* and the effects of individual selected factors can be studied.

Human primary cells are a natural *in vitro* experimental model. However, the establishment of human muscle cell cultures involves obtaining a muscle sample by an invasive biopsy technique and a limited amount of material. Therefore non-human cell lines are often used as an alternative. Moreover, compared to primary cells, cell lines are well characterized and not influenced by donor-to-donor variability. They are easily obtained, homogenous and inexhaustible experimental material.

We used primary human skeletal muscle cells and C2C12 mouse myoblast cell line in our experiments. In both models, proliferating myoblasts are able to differentiate into post-mitotic multinucleated myotubes that acquire a muscle-specific phenotype [196, 197]. We used both undifferentiated myoblasts and differentiated myotubes, which serve as an *in vitro* model of muscle fibers. Myoblasts are derived from satellite cells and play an important role in growth, repair and regeneration of skeletal muscle [198].

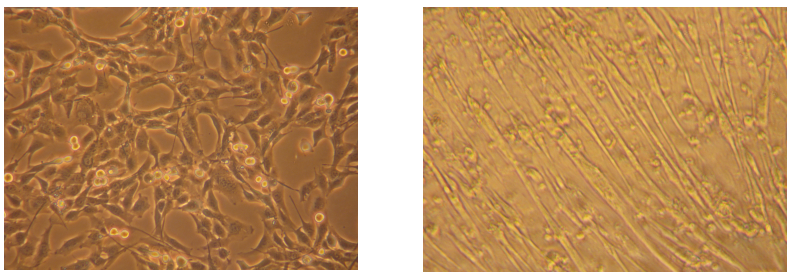

**Figure 6:** Images of a monolayer of undifferentiated, mononuclear C2C12 myoblasts (left) and differentiated, multinucleated C2C12 myotubes (right).

In addition, we used a vegan population as a model to study the effects of chronic changes in nutritional habits. Vegans consume a strict plant-based diet and the proportion and composition of nutrients in their diet is therefore different from a conventional diet [199].

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

Excess of FFA and 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 [128, 161, 168] which can eventually lead to a cell death [168, 180]. 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 (see chapter 1.2.2.4). 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. PA was selected because it is the most common saturated FFA in plasma and tissues of mammals [43, 200].

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 [168, 201]. We used 100 μM concentration of PA, which we considered as a physiologically relevant dose [202]. 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 as it reflects the maximum ability of mitochondria to maintain energy production in response to an increase in energy demand, like in conditions of acute or chronic stress [203, 204].

Previous studies reported protective effects of MitoQ and MitoTEMPOL against mitochondrial oxidative damage in various cell types [205-207]. 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. The lack of protection in our study could be explained e.g. by a dependence of protective effects on cell type, number of mitochondria in cells or the degree of reliance on oxidative phosphorylation. 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 [208]. These findings indicate that TPP-conjugated antioxidants should be used carefully and their potential effects on cellular bioenergetics should be taken into account. Cells exposed to TPP moiety should always be included as a control group.

In summary, our results suggest that skeletal muscle myoblasts (progenitor cells) may be the first target of the deleterious action of PA, as they showed to be more sensitive to its effects then myotubes. PA reduced mitochondrial respiration of myoblasts and decreased their viability. Mitochondria-targeted antioxidants

were unable to prevent PA-induced cell death in myoblasts and they markedly inhibited mitochondrial respiration. These results are presented in Article 1.

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 [128, 160, 177, 178]. However, understanding of the mechanisms involved is still incomplete. FFA are endogenous ligands of PPAR, transcription factors regulating the expression of genes involved in lipid and energy metabolism [190]. 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. We observed this effect even for high total concentration of FFA (1000 μM) suggesting an efficient mechanism by which unsaturated FFA protect cells against FFA load. 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. In contrary, saturated PA was a weak activator of PPARδ as was previously suggested [185]. 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. Moreover, PPARδ agonist did not prevent cytotoxic effect of PA suggesting that this effect is not mediated by a lack of PPARδ activation.

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 AMPK-dependent mechanism, without an involvement of PPAR [192]. The protective effect of unsaturated FFA may also involve the channeling of PA into TAG, as activation of this pathway decreases the level of free intracellular fatty acids. This mechanism was reported in cultured muscle cells for oleic and arachidonic acid [128, 180, 209].

FFA are known uncouplers of mitochondrial respiration but the specificity of different types of FFA is less clear [210]. In our study, 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 [211]. Similar uncoupling effect was observed also for agonist GW501516.

Taken together, we found that unsaturated FFA are effective ligands of PPARδ, however, PPARδ activation is not involved in their protective effects against PA-induced lipotoxicity in C2C12 myotubes. Unsaturated FFA, but not saturated PA, caused mild uncoupling of mitochondrial respiration. These results are presented in Article 2.

### 3.2.3 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 [102] (see chapter 1.2.2.1). 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 [199, 212, 213], and also a lower IMCL content [199], compared with omnivores. However, the causes of these metabolic differences are not clear. Different composition of consumed fat has been suggested to play a role [199]. We hypothesized that a higher mitochondrial density and changes of IMCL content in skeletal muscle may be involved. 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 and muscle FFA pools 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). The composition of muscle FFA pool, 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 [199, 212]. 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* [133, 214]. Therefore, these parameters should be a focus of a future research. 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 [114, 147] (see chapter 1.2.2.3).

Vegans were reported to have a higher intake of polyunsaturated fat, and lower intake of saturated fat [199, 215]. 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 [199, 216]. On the other hand, circulating EPA and DHA levels were shown to be lower in vegans due to their lower dietary intake [217]. These n-3 PUFA are also referred to as marine fatty acids as they are abundant in oily fish and their consumption was shown to have a myriad of metabolic benefits [218]. We found that vegans had lower DHA in skeletal muscles compared with omnivores, however, clinical impact of this observation deserves further investigation.

In conclusion, we found that metabolic benefits, such as lower fasting plasma glucose and higher insulin-stimulated glucose uptake, in vegans are not associated with alterations in IMCL content or changes in mitochondrial density in skeletal muscle. Plasma and muscle FFA profiles showed a specific pattern, whose clinical impact needs to be further elucidated. These results are presented in Article 3.

### 3.2.4 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 (see chapter 1.1.2.4). It has been used clinically as a nutrition supplement in a wide range of wasting diseases [74], which are characterized by low muscle and plasma glutamine concentrations [78, 79]. In cell cultures, glutamine is required for cell growth [84, 85] and proliferating cells use glutamine as both nitrogen donor and energy substrate [86, 219]. 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 [84, 85], 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 [220] 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. Considering the fact that muscle mass regeneration during recovery from wasting illnesses is dependent on the function of myoblasts [198], these results suggest that increasing glutamine concentration to normal levels may not improve conditions for muscle regeneration. However, extrapolating *in vitro* data to whole-body physiology is certainly difficult.

The most interesting finding from mitochondrial respiration analysis in myoblasts and myotubes is the uncoupling effect of high doses of glutamine. Highly coupled respiration with ~90% of basal respiration being used to drive ATP synthesis 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 [221] and to increase energy expenditure and fat oxidation in healthy subjects [222]. 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 [223]. If the mitochondrial uncoupling is due to increased nonspecific proton leak or controlled leak through uncoupling proteins 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 (300 μM) represent optimal condition for myoblast proliferation and for efficiency of aerobic phosphorylation in an *in vitro* model of human skeletal muscle. These results are presented in Article 4.

# ORIGINAL ARTICLES

**Article 1**

Jana Patková, Michal Anděl and Jan Trnka

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

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**Original Paper**

# Palmitate-Induced Cell Death and Mitochondrial Respiratory Dysfunction in Myoblasts are Not Prevented by Mitochondria-Targeted Antioxidants

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**Key Words**
Palmitate • Mitochondrial respiration • mtDNA integrity • Mitochondria-targeted antioxidants • MitoQ • MitoTEMPOL • Skeletal muscle • Myoblasts • Myotubes • Spare respiratory capacity

**Abstract**
***Background/Aims:*** Deleterious effects of saturated fatty acids in skeletal muscle cells are well known but their impact on mitochondrial respiration has not been well studied. Mitochondrial oxidative damage has been implicated to play a role in their effect. The purpose of this study was to evaluate viability, mtDNA integrity and mitochondrial respiration in C2C12 myoblasts and myotubes exposed to palmitate and to test the effect of mitochondria-targeted antioxidants MitoQ and MitoTEMPOL in preventing palmitate-induced damage. ***Methods:*** Cells were treated with tested compounds, mtDNA damage was detected by quantitative PCR and mitochondrial respiration was measured using an extracellular flux analyzer XF24. ***Results:*** Palmitate caused mtDNA damage, which was associated with reduced mitochondrial respiration and cell death in myoblasts but not in myotubes. MitoTEMPOL was able to prevent palmitate-induced mtDNA damage in myoblasts but failed to prevent cell death. MitoQ did not show any protective effect and both compounds markedly inhibited mitochondrial respiration. ***Conclusion:*** Our results indicate that skeletal muscle progenitor cells could be the first target of the deleterious action of palmitate, as myoblasts appeared to be more sensitive to its effects than myotubes possibly in part due to a lower spare respiratory capacity in the former. Only MitoTEMPOL prevented palmitate-induced mtDNA damage but neither antioxidant was able to prevent cell death and both antioxidants had a marked negative effect on respiration.



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## Introduction

Elevated levels of plasma free fatty acids (FFA), which are characteristic for obesity and type 2 diabetes [1, 2], are associated with various pathophysiological consequences in skeletal muscle, although recently this simple association has been put into question [3]. Development of insulin resistance and changes in mitochondrial function in skeletal muscle have been found in healthy humans and animals after lipid infusion [4, 5] or after prolonged fasting [6]. Experiments on cultured skeletal muscle cells show that exposure to long-chain saturated FFA but not unsaturated FFA leads to the induction of insulin resistance, inflammation, some features of mitochondrial dysfunction and apoptosis [7-9]. Although information has accumulated regarding the role of FFA in the induction of insulin resistance in skeletal muscle and cultured skeletal muscle cells [4, 5, 7, 10] less data is available about their effect on mitochondrial function and specifically mitochondrial respiration.

Metabolic states characterized by hyperglycemia and high levels of circulating free fatty acids have been shown to be associated with a chronic oxidative stress and increased mitochondrial reactive oxygen species (ROS) production [11, 12]. Increased production of hydrogen peroxide associated with insulin resistance was observed also in rodents on high fat diet with no change in mitochondrial respiratory function [13]. There are several studies in cultured skeletal muscle cells showing an increase of mitochondrial ROS production induced by palmitate [14-16] accompanied by mtDNA damage and some features of mitochondrial dysfunction [17, 18]. Mitochondrial DNA has an important role in the regulation of mitochondrial respiration and oxidative damage of mtDNA was postulated as an initial event leading to mitochondrial dysfunction [18].

Therapy that can decrease mitochondrial oxidative damage could prevent damage associated with excess of nutrients such as saturated fatty acids. Mitochondria-targeted antioxidants accumulate in mitochondria and have been shown to be more effective in preventing mitochondrial oxidative damage than untargeted antioxidants [19, 20]. MitoQ and MitoTEMPOL are two promising compounds, whose targeting to mitochondria is achieved by the covalent attachment of an antioxidant molecule to the lipophilic triphenylphosphonium cation ($TPP^+$) [21]. The shielded positive charge of these lipophilic cations enables them to permeate lipid bilayers easily and to accumulate several hundred-fold within mitochondria, due to the large membrane potential [22]. MitoQ is a ubiquinone derivative which exhibited both antioxidant and anti-apoptotic properties in a large number of *in vitro* as well as *in vivo* studies [19, 23-26]. MitoTEMPOL consists of the piperidine nitroxide TEMPOL conjugated with a $TPP^+$ moiety. It has been shown to be effective in preventing lipid peroxidation and mtDNA damage in skeletal muscle cells [27] or in protecting pancreatic β-cells against oxidative stress under glucolipotoxic conditions [24].

The purpose of the present study was to clarify the effect of palmitate on viability, mtDNA integrity and mitochondrial respiration in C2C12 skeletal muscle cells. We tested the effect of palmitate both on differentiated myotubes and undifferentiated myoblasts. We found palmitate-induced mtDNA damage which was associated with reduced mitochondrial respiration, increased production of hydrogen peroxide and cell death in myoblasts, but not in myotubes. Following on the hypothesis that palmitate caused damage through an induction of oxidative stress, we hypothesized that treatment with mitochondria-targeted antioxidants might prevent this damage in myoblasts and we tested two such antioxidants, MitoQ and MitoTEMPOL.

## Materials and Methods

*Materials*

Mitochondria-targeted antioxidants MitoQ and MitoTEMPOL were kindly provided by Dr. Michael P. Murphy (MRC Mitochondrial Biology Unit, Cambridge, UK). Propyltriphenylphosphonium bromide, palmitate and bovine serum albumin (fatty acid free) were purchased from Sigma-Aldrich. Fetal bovine

serum for cell cultures was from Life Technologies, penicillin and streptomycin were from Sigma-Aldrich. Blood & Cell Culture DNA Mini Kit for DNA isolation was obtained from Qiagen and reagents for QPCR from Applied Biosystems.

*Cell culture and treatment*

As a model of skeletal muscle we used murine C2C12 myoblasts. Cells were obtained from the European Collection of Cell Cultures and were used as myoblasts, which served as a model of satellite cells, and as myotubes, an *in vitro* model of muscle fibres. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in an atmosphere of 95% humidity and 5% $CO_2$ and maintained below a maximal confluence of 70-80%. To initiate differentiation, cells were allowed to reach 100% confluence and the medium was changed to the differentiation medium (DMEM containing 2% fetal bovine serum), which was replaced every 48 h. Myotubes were used for experiments after 7 days, when full differentiation was observed.

A stock concentration of palmitate was prepared by conjugating palmitate with fatty acid-free bovine serum albumin (BSA), using a modified method described by Cousin [28]. Briefly, palmitate was dissolved in 0.1 M sodium hydroxide at 70°C to a final concentration of 100 mM. Palmitate was then mixed with 15% fatty acid-free BSA at 50°C for 30 min, yielding a final stock solution of 5 mM. The molar ratio of FFA/BSA was 2:1 in this preparation which is close to the ratio observed in human serum [29]. A control BSA solution was prepared by mixing a volume of 0.1 M sodium hydroxide equal to that of palmitate in the palmitate-BSA solution with 15% fatty acid-free BSA. The pH of both solutions was adjusted to 7.4. After sterilization by filtration the actual concentration of palmitate was determined using the NEFA (non-esterified fatty acids) kit (Randox Laboratories, UK).

Cells were incubated for 18 h in DMEM containing palmitate-BSA conjugate or DMEM with BSA only (control). For experiments with antioxidants MitoQ or MitoTEMPOL were added to cells 1 h prior to the addition of palmitate and then incubated for 18 h. All compounds were washed away before measurements. We also treated cells with corresponding concentrations of propylTPP (pTPP) to control for the effect of the cation moiety itself [30].

*Cell viability assay*

Cell viability after the exposure to various concentrations of palmitate and antioxidants was assessed using the MTS assay (Promega). The incubation medium with tested compounds was changed to a medium with the MTS reagent at the end of exposure and cells were incubated for another 2 h at 37°C. The absorbance of dissolved formazan was measured at 490 nm in a microplate reader. Data are displayed as a percentage of cells with no additions.

*Detection of extracellular hydrogen peroxide*

Extracellular hydrogen peroxide ($H_2O_2$) was measured using the Amplex Red reagent (Life Technologies) by monitoring its oxidation to fluorescent resorufin in the presence of horseradish peroxidase (HRP). Cells were grown in 96-well plates and treated as described above. The incubation medium was then removed and cells were incubated with the reaction mixture at 37°C for 30 min protected from light. The reaction mixture consisted of 50 μM Amplex Red reagent and 0.1 U/ml HRP in Krebs-Ringer phosphate (145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM glucose, pH 7.35). Fluorescence was detected at 590 nm with excitation at 540 nm. Protein content in each well was then determined by the bicinchoninic acid kit (Sigma). Data are expressed as relative fluorescence units (RFU) of resorufin ($H_2O_2$ emission) per microgram of protein.

*Analysis of mtDNA damage by quantitative PCR*

The integrity of mtDNA was assessed following treatments, using the quantitative polymerase chain reaction (QPCR) methodology [31, 32] with some modifications. The QPCR assay is based on the fact that any damage to DNA blocks the progression of the DNA polymerase on a template and will result in a decreased amplification. Amplification is thus inversely proportional to the amount of DNA damage on a given template.

Cells were incubated with tested compounds in 6 cm Petri dishes and high molecular weight DNA was then isolated with the Blood & Cell Culture DNA Mini Kit (Qiagen) as described in the manufacturer's protocol with some modifications. The tissue protocol was used, since the protocol for DNA extraction of cultured cells involves an isolation of nuclei and hence a loss of mtDNA [31]. The concentration of total cellular DNA was determined fluorometrically using Qubit dsDNA HS Assay Kit and Qubit fluorometer (Life Technologies). Stock DNA was diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to a final concentration of 3 μg/ml, which was used as a template for PCR. Two targets of mtDNA were amplified: a long target to quantify levels of mtDNA lesions and a short target to control for changes in mtDNA copy number. The long target (≈ 10 kbp) spanned ND1 and ND5 genes and the small target (127 bp) was part of ND1 gene. The primers were synthesized by Sigma and the primer nucleotide sequences were as follows: 5'-GCC AGC CTG ACC CAT AGC CAT AAT-3' and 5'-GCC GGC TGC GTA TTC TAC GTT A-3' for short target, 5'-GCC AGC CTG ACC CAT AGC CAT AAT-3' and 5'-GAG AGA TTT TAT GGG TGT AAT GCG G-3' for long target. The total volume for each PCR reaction was 20 μl, consisting of 15 ng of DNA template, 1 U rTth DNA polymerase XL (Applied Biosystems), 1× buffer XL (provided with the rTth polymerase), 200 μM dNTPs, 20 pmol forward primer, 20 pmol reverse primer, 100 μg/ml of BSA, 1 mM $Mg(OAc)_2$ for long target and 1.5 mM $Mg(OAc)_2$ for short target. To ensure quantitative conditions, a control reaction containing 50% of template DNA was included during each set of amplification and only amplification ranging from 40 to 60% was considered acceptable. Automated hot-start using AmpliWax PCR Gems 50 (Applied Biosystems) was used to increase sensitivity and specificity of PCR. The parameters for the short mitochondrial target were 1 min at 94°C, then 20 cycles of 30 s at 94°C, 45 s at 64°C, 45 s at 72°C, followed by a final extension step of 10 min at 72°C. The parameters for the long mitochondrial target were 1 min at 94°C, then 18 cycles of 15 s at 94°C, 12 min at 64°C, followed by a final extension step of 10 min at 72°C. The PCR products were then quantified fluorometrically and the results were expressed as amplification relative to the control, which was defined as undamaged for the purposes of this assay.

*Analysis of mitochondrial respiration*

Oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured using an extracellular flux analyzer XF24 (Seahorse Bioscience). The assay medium consisted of modified DMEM lacking sodium bicarbonate (XF Assay medium, Seahorse Bioscience) supplemented with 4 mM L-glutamine, 1 mM pyruvate and 5.5 mM glucose. The pH was adjusted to 7.4 at 37°C on the day of the experiment.

For experiments with myoblasts cells were seeded at a density of 13,000 cells per well in 24-well plates designed for this instrument and left to attach for at least 2 h. Cells were then treated with tested compounds as described above. For experiments with myotubes cells were differentiated for 7 days in assay plates prior to treatment. The culture medium was replaced with the assay medium 1 h prior to measurement and cells were kept at 37 °C in an atmosphere without $CO_2$. Mitochondrial stress test was then performed to assess the bioenergetic status of the cells [33]. OCR measurements were obtained before and after sequential additions of the ATPase inhibitor oligomycin (1 μM), inner membrane uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 1 μM) and inhibitors of complex I and III rotenone (1 μM) and antimycin A (1 μM) in myoblasts. For myotubes the concentration of FCCP was increased to 1.5 μM to obtain a maximal effect. After completion of the measurement, assay medium was removed and well contents were lysed in CelLytic MT lysis buffer (Sigma). Protein content in each well was determined by bicinchoninic acid kit (Sigma). The following parameters were then calculated: basal respiration (baseline respiration before addition of compounds), ATP turnover-linked respiration (oligomycin-sensitive respiration), maximal respiration (maximal uncoupled respiration), spare respiratory capacity (maximal uncoupled respiration minus basal respiration) and proton leak (oligomycin-insensitive respiration). Basal respiration is controlled strongly by ATP turnover, which alters mainly in response to ATP demand in the cell, and by changes in proton leak [34]. Maximal respiration after the addition of an uncoupler then reflects the maximal capacity of the electron transport chain. Non-mitochondrial respiration (after the addition of rotenone/antimycin A) was subtracted from basal and maximal respiration and proton leak. Data are expressed as pmol $O_2$/min/μg protein. Coupling efficiency (CE) was calculated as the ratio of ATP turnover-linked respiration and basal respiration [33].

Glycolytic activity (ECAR) was measured in parallel with respiration and the following parameters were calculated: basal ECAR (before addition of compounds), oligomycin-stimulated ECAR, and glycolytic reserve capacity (oligomycin-stimulated minus basal ECAR). Data are expressed as mpH/min/μg protein.

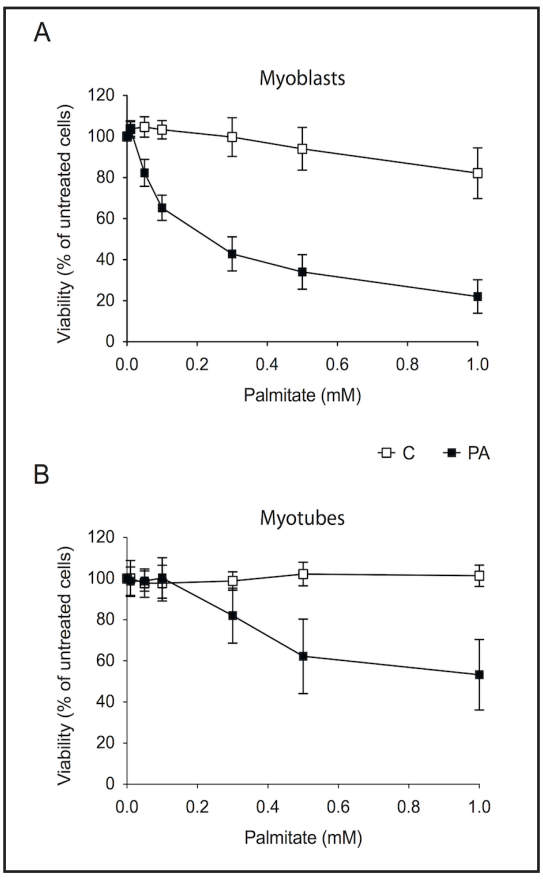


**Fig. 1.** Palmitate decreased viability of myoblasts (A) and myotubes (B) in a dose-dependent manner. Cells were treated with palmitate (PA) in concentrations varying from 0.01 to 1 mM or corresponding concentrations of BSA (C) for 18 h and cell viability was assessed using MTS assay. The effect of palmitate was statistically significant from 0.05 mM PA in myoblasts and 0.3 mM PA in myotubes. Only the highest concentration of BSA (corresponding to 1 mM palmitate) significantly decreased viability of myoblasts. Data are displayed as a percentage of untreated cells and presented as means and 95% CI from five independent experiments.

*Citrate synthase activity measurement*

The citrate synthase activity was determined in whole cell extracts using the Citrate Synthase Assay Kit (Sigma). The assay mixture contained 0.3 mM acetyl coenzyme A, 0.5 mM oxaloacetate and 0.1 mM 5,5-dithiobis(2-nitrobenzoic acid). Protein content of samples was determined by the bicinchoninic acid kit. The enzyme activity was measured spectrophotometrically at 30°C at 412 nm and expressed in nmol/min/mg protein.

*Statistical analysis*

All data represent at least three experiments and are expressed as means and 95% confidence intervals (CI, in square brackets). Mixed-effects model ANOVA with treatment as the fixed effect and set of experiment as the random effect was used followed by Dunnett post-hoc test for multiple comparisons. All analyses were performed in Statistica 9.1 (StatSoft, Inc.). P values less then 0.05 were considered statistically significant.

## Results

*Palmitate decreased cell viability and caused mtDNA damage in myoblasts and myotubes*

We evaluated the cytotoxic effect of palmitate on myoblasts and myotubes in the concentration range of 0.01 - 1 mM. Cells were exposed to palmitate-BSA conjugate or to corresponding concentrations of BSA only. The results of MTS assays show a dose-dependent reduction of viability in both myoblasts and myotubes after an 18 h exposure to palmitate (Fig.1A and B). Myoblasts showed a higher sensitivity to the cytotoxic effect of palmitate than differentiated myotubes. Only the highest concentration of BSA (corresponding to 1 mM palmitate) led to significantly decreased viability of myoblasts (Fig. 1A).

Based on toxicity assays 100 μM concentration of palmitate was chosen and its effect on mtDNA integrity was tested. We consider this concentration of palmitate as physiologically relevant: although total plasma fatty acids can be as high as 1 mM or even higher under some physiological and pathological conditions (prolonged fasting, obesity, type 2 diabetes) [4], the concentration of palmitate under most conditions is generally much lower [35]. We used the QPCR assay to monitor damage to mtDNA by comparing the relative amplification of a very long fragment of mtDNA from treated and non-treated samples. As shown in Fig. 2A the relative amplification of the long mtDNA fragment was decreased both in myoblasts (difference of means C vs. PA -15.18 [-27.78, -2.59]) and myotubes (-16.84 [-29.68, -4.01]) after palmitate exposure suggesting that palmitate caused similar mtDNA damage.

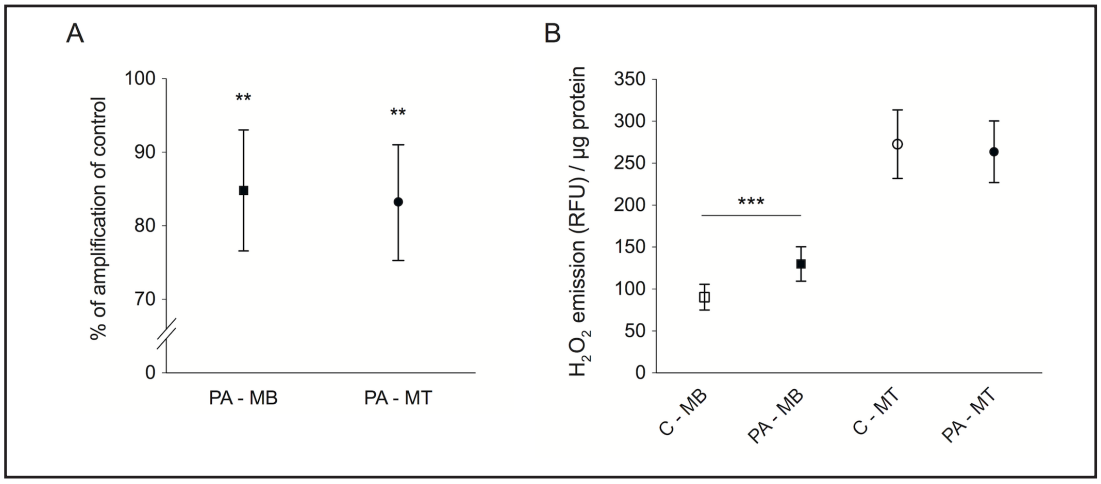


**Fig. 2.** Palmitate-induced mtDNA damage in myoblasts (PA-MB) and myotubes (PA-MT) and increased $H_2O_2$ production in myoblasts. Cells were treated with 100 µM palmitate or 0.3% BSA for 18 h. A) integrity of mtDNA was assessed by QPCR, where mtDNA of controls was defined as undamaged (100% amplification). Palmitate significantly decreased relative amplification of 10 kb fragment of mtDNA both in myoblasts and myotubes. B) extracellular $H_2O_2$ was measured by monitoring Amplex Red oxidation to fluorescent resorufin. Palmitate significantly increased $H_2O_2$ production only in myoblasts. Results are presented as means and 95% CI from at least four independent experiments. ** $p < 0.01$, *** $p < 0.001$ compared to controls.

*Palmitate increased reactive oxygen species (ROS) production in myoblasts but not in myotubes*

Extracellular hydrogen peroxide was measured to evaluate the role of ROS in the effect of palmitate. As shown in Fig. 2B myoblasts exposed to 100 µM palmitate showed significantly higher hydrogen peroxide production compared to controls (difference of means 39.54 [15.10, 63.97] RFU/µg protein). Myotubes showed higher basal levels of hydrogen peroxide in controls, which were not further increased after the treatment with palmitate (-9.03 [-60.73, 42.66]).

*Palmitate affected mitochondrial respiration more in myoblasts than in myotubes*

After treatment with 100 µM palmitate for 18 h, the bioenergetic status of cells was assessed as shown in Fig. 3A. Palmitate exposure led to a changed bioenergetic profile of both myoblasts and myotubes but in a different manner, as shown in Fig. 3B and C. In myoblasts palmitate exposure led to a decreased basal respiration (difference of means -2.88 [-5.98, 0.22] pmol $O_2$/min/µg protein), ATP turnover-linked respiration (-2.86 [-5.47, -0.25]), maximal respiration (-10.59 [-17.68, -3.50]) and spare respiratory capacity (SRC) (-7.71 [-11.94, -3.47]) compared to controls while there was virtually no difference in proton leak (-0.02 [-0.58, 0.53], Fig. 3B).

In myotubes exposed to palmitate basal respiration was significantly increased compared to controls (difference of means 1.16 [0.15, 2.16]) due to a higher proton leak (0.60 [0.31, 0.90]) but other parameters were not significantly affected (difference of means for ATP-turnover linked respiration 0.55 [-0.22, 1.32], maximal respiration 2.44 [-2.74, 7.62] and SRC 1.29 [-3.25, 5.82], Fig. 3C). We found a decrease in coupling efficiency (CE) in both myoblasts (difference of means C vs. PA -0.04 [-0.06, -0.02]) and myotubes (-0.05 [-0.06, -0.03]) exposed to palmitate (Fig. 3D). There was no difference in non-mitochondrial respiration between palmitate-treated and control cells in either group (data not shown). Extracellular acidification rates did not differ significantly between palmitate-treated and control myoblasts or myotubes suggesting no difference in glycolytic activity (Table 1).

*Citrate synthase activity was decreased in palmitate-treated myoblasts but not in myotubes*

Citrate synthase (CS) activity was measured as a mitochondrial mass marker. CS activity decreased significantly in myoblasts after the treatment with palmitate (difference of means

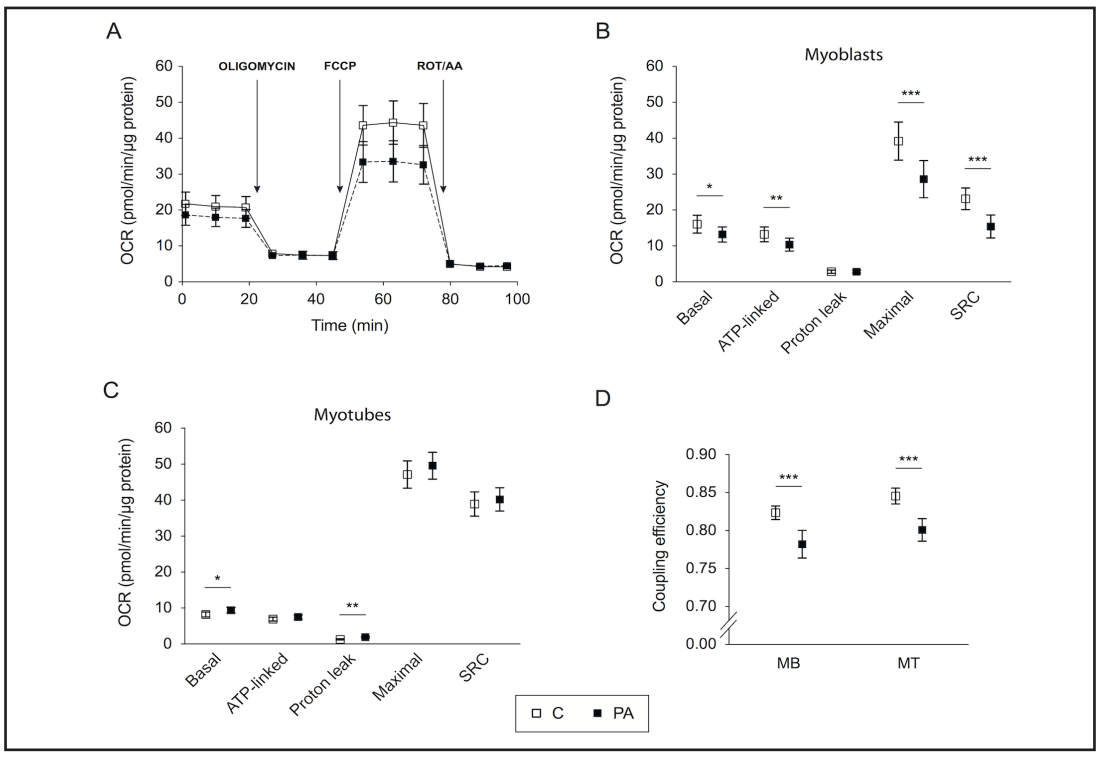


**Fig. 3.** Effect of palmitate exposure on mitochondrial respiration (oxygen consumption rates, OCR) in myoblasts and myotubes measured under different respiratory conditions. Cells were treated with 100 µM palmitate (PA) or 0.3% BSA (C) for 18 h and then the growth media with palmitate was removed and replaced with assay media 1 h prior the assessment of mitochondrial function. OCR was measured before and after sequential injections of the indicated compounds. A) mitochondrial stress test experiment showing times of mitochondrial inhibitors addition to myoblasts. B,C) individual mitochondrial respiratory parameters (basal, ATP turnover-linked and maximal respiration; proton leak and spare respiratory capacity - SRC) calculated for myoblasts (B) and myotubes (C) as described in methods section. D) palmitate-induced decrease in coupling efficiency both in myoblasts (MB) and myotubes (MT). Results are presented as means and 95% CI from four independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to controls.

**Table 1.** Glycolytic activity (ECAR) in myoblasts and myotubes after the treatment with palmitate. Data are expressed as mpH/min/µg protein and results are presented as means and 95% CI from four independent experiments

| | Basal | Oligomycin-stimulated | Glycolytic reserve capacity |
|---|---|---|---|
| Myoblasts | | | |
| Control | 1.83 [1.44, 2.23] | 2.85 [2.26, 3.44]] | 1.02 [0.78, 1.25] |
| PA | 2.04 [1.77, 2.32] | 3.16 [2.69, 3.62] | 1.11 [0.90, 1.33] |
| Myotubes | | | |
| Control | 0.38 [0.29, 0.48] | 0.73 [0.60, 0.87] | 0.35 [0.29, 0.41] |
| PA | 0.43 [0.30, 0.55] | 0.74 [0.56, 0.92] | 0.32 [0.24, 0.39] |

C vs. PA -15.63 [-29.97, -1.30] nmol/min/mg protein), whereas no significant difference was observed between palmitate-treated and control myotubes (-1.44 [-39.45, 36.57], Table 2).

Since the effects of palmitate on respiratory and mitochondrial parameters in myotubes were negligible further experiments using mitochondria-targeted antioxidants were performed only in myoblasts.

**Table 2.** Citrate synthase activity in myoblasts and myotubes after the treatment with palmitate. Data are expressed as nmol/min/mg protein and results are presented as means and 95% CI from four independent experiments. * $p < 0.05$ compared to controls

| | Control | PA |
|---|---|---|
| Myoblasts | 147.36 [135.86, 158.87] | 131.73 [122.08, 141.37] * |
| Myotubes | 283.77 [255.66, 311.88] | 282.33 [253.40, 311.26] |

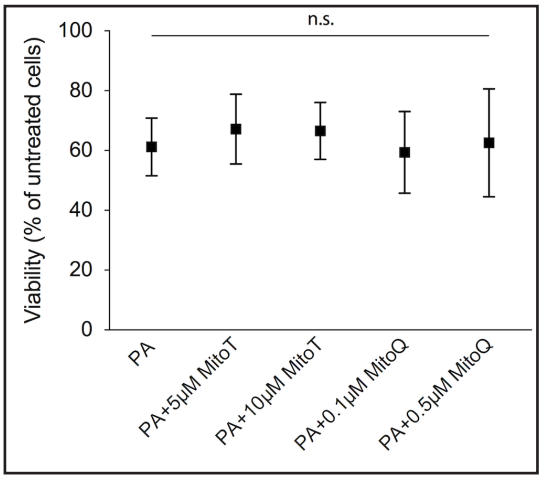


**Fig. 4.** Neither MitoQ nor MitoTEMPOL prevented palmitate-induced cell death in myoblasts. Cells were pre-treated with MitoQ or MitoTEMPOL at the indicated concentrations for 1 h and then co-incubated with palmitate for 18 h. Cell viability was then assessed using MTS assay. Data are displayed as a percentage of untreated cells. Results are presented as means and 95%CI from four independent experiments, n.s. – not significantly different from palmitate.

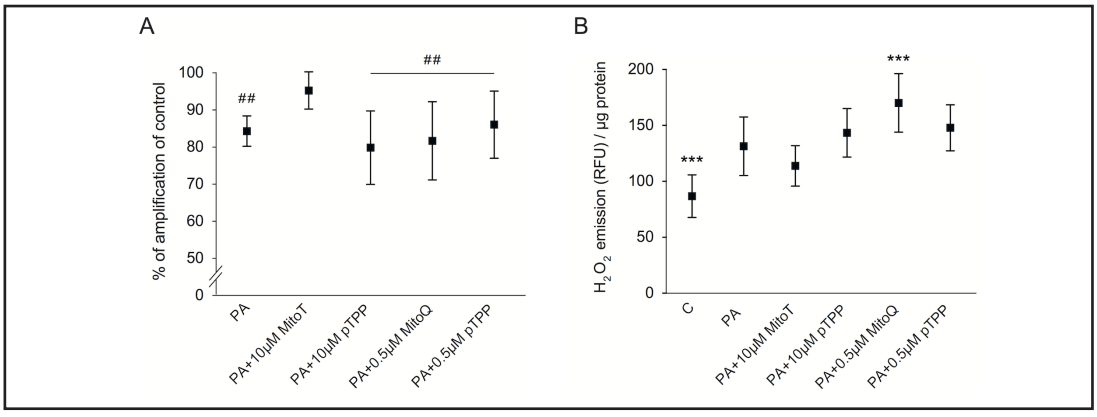


**Fig. 5.** MitoTEMPOL but not MitoQ decreased palmitate-induced mtDNA damage and hydrogen peroxide production in myoblasts. Cells were pre-treated with 0.5 μM MitoQ, 10 μM MitoTEMPOL or corresponding concentrations of pTPP for 1 h and then co-incubated with palmitate for 18 h. A) integrity of mtDNA was assessed by QPCR. MitoTEMPOL prevented a palmitate-induced decrease in the relative amplification of 10 kb fragment of mtDNA. B) extracellular $H_2O_2$ was measured by monitoring Amplex Red oxidation to fluorescent resorufin. MitoTEMPOL decreased palmitate-induced $H_2O_2$ production but the difference did not reach statistical significance. MitoQ treatment led to a significantly increased $H_2O_2$ production. Results are presented as means and 95%CI from three (A) or four (B) independent experiments. ## $p < 0.01$ compared to controls, *** $p < 0.001$ compared to palmitate.

*Mitochondria-targeted antioxidants had no effect on palmitate cytotoxicity*

If antioxidants are to be used in cells it is important to ensure that doses used are not toxic to a given cell type. We tested the cytotoxicity of both antioxidants in myoblasts in a broad concentration range. MitoQ significantly decreased cell viability at concentrations over 0.5 μM and MitoTEMPOL for concentrations over 10 μM (data not shown). Based on these results we pre-treated cells with 0.1 or 0.5 μM MitoQ, 5 or 10 μM MitoTEMPOL or

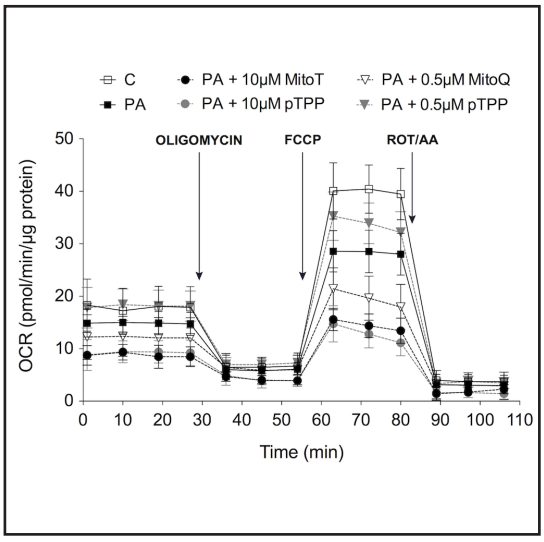


**Fig. 6.** Effect of MitoQ, MitoTEMPOL and pTPP compounds on palmitate-affected mitochondrial respiration of myoblasts. Cells were pre-treated with 0.5 μM MitoQ, 10 μM MitoTEMPOL or corresponding concentrations of pTPP for 1h and then co-incubated with palmitate for 18 h. The growth medium with tested compounds was removed and replaced with the assay medium 1 h prior the assessment of mitochondrial function. Oxygen consumption rates (OCR) were measured before and after sequential injections of the indicated compounds. Both MitoQ and MitoTEMPOL markedly inhibited mitochondrial respiration of myoblasts compared to controls or cells exposed only to palmitate. The effect was observed also for higher concentration of pTPP (10 μM). Results are presented as means and 95%CI from three independent experiments.

corresponding concentrations of propylTPP (pTPP) for 1 h and then co-incubated with palmitate for 18 h. We found no difference in the viability of cells treated only with palmitate and cells pre-treated with two different concentrations of MitoQ, MitoTEMPOL or pTPP (Fig. 4, data for pTPP not shown). Mitochondria-targeted antioxidants MitoQ and MitoTEMPOL were therefore unable to prevent palmitate-induced cell death.

*MitoTEMPOL but not MitoQ prevented palmitate-induced mtDNA damage and both antioxidants inhibited mitochondrial respiration*

Based on toxicity assays and the literature [23, 27, 36, 37] we used 0.5 μM concentration of MitoQ and 10 μM concentration of MitoTEMPOL for subsequent experiments testing their ability to prevent palmitate-induced mtDNA damage and mitochondrial respiratory dysfunction. Treatment with MitoTEMPOL prevented palmitate-induced mtDNA damage as shown by the relative amplification of the long mtDNA fragment (difference of means C vs. PA -15.67 [-28.24, -3.11], C vs. PA + 10 μM MitoTEMPOL -4.72 [-17.29, 7.84], Fig. 5A). No effect was observed for MitoQ and for either concentration of pTPP (difference of means C vs. PA + 0.5 μM MitoQ -18.31 [-31.27, -5.36], C vs. PA + 10 μM pTPP -20.14 [-32.71, -7.57], C vs. PA + 0.5 μM pTPP -13.93 [-26.50, -1.37], Fig. 5A).

Both antioxidants had a profound negative effect on mitochondrial respiration (Fig. 6). They markedly inhibited basal respiration, ATP turnover-linked respiration, maximal respiration and spare respiratory capacity compared to controls or cells exposed only to palmitate. Only proton leak and non-mitochondrial respiration were not affected. Inhibition of respiration was not exclusive for antioxidants themselves but was observed also for the 10 μM concentration of pTPP pointing to a deleterious effect of the cations themselves.

*The effect of MitoQ and MitoTEMPOL on reactive oxygen species production in palmitate-treated myoblasts*

MitoTEMPOL had a promising antioxidant potential as it significantly decreased hydrogen peroxide production in non-treated control myoblasts (difference of means C vs. 10 μM MitoTEMPOL -22.1 [-41.54, -2.647] RFU/μg protein, data not shown). In myoblasts exposed to palmitate MitoTEMPOL decreased palmitate-induced production of hydrogen peroxide but the difference did not reach statistical significance (C vs. PA 44.62 [8.13, 81.11], PA vs. PA + 10 μM MitoTEMPOL -17.52 [-54.03, 18.99], Fig. 5B). MitoQ did not prevent palmitate-induced production of hydrogen peroxide and caused even increased production of this ROS (PA vs. PA + 0.5 μM MitoQ 38.76 [2.23, 75.25], Fig. 5B). This increase was observed

also in non-treated control cells exposed to MitoQ (C vs. 0.5 μM MitoQ 38.97 [15.12, 62.82], data not shown). Control pTPP compounds only slightly increased the production of ROS in palmitate-treated myoblasts (not statistically significant, PA vs. PA + 10 μM pTPP 12.08 [-24.41, 48.57], PA vs. PA + 0.5 μM pTPP 16.48 [-20.83, 53.79], Fig. 5B).

## Discussion

Increased oxidative stress associated with some features of mitochondrial dysfunction was reported in skeletal muscle cells after the exposure to palmitate [17, 18]. However, the effects of saturated fatty acids on mitochondrial respiration have not been well studied, particularly in undifferentiated skeletal muscle cells. The present study was designed to clarify the effect of palmitate on viability and mitochondrial respiratory function in differentiated and undifferentiated C2C12 skeletal muscle cells and to test the ability of mitochondria-targeted antioxidants MitoQ and MitoTEMPOL to prevent palmitate-induced damage.

Palmitate appeared to be cytotoxic for myoblasts as well as myotubes in a dose-dependent manner although myoblasts were much more sensitive. We have chosen a physiologically relevant concentration of palmitate (100 μM) [35] to study further whether mtDNA integrity is affected in palmitate-treated cells. Mitochondrial DNA is one of the main targets for reactive oxygen species and its damage has been suggested as a marker of oxidative damage [38]. We found mtDNA damage after the treatment with palmitate in both myoblasts and myotubes using the QPCR method, which is able to detect a wide range of types of DNA lesions. Our observation is in accordance with another study [18] where mtDNA damage was detected in L6 myotubes exposed to palmitate using quantitative alkaline Southern blot analysis. Authors of this study postulated that mtDNA damage is a critical event leading to mitochondrial dysfunction with consequent increase in oxidative stress and finally to apoptosis [18]. In our experiments this was true only for myoblasts, as mtDNA damage after the exposure to palmitate was similar in myoblasts and myotubes but we detected mitochondrial dysfunction (discussed below) with increased hydrogen peroxide production (by about 50%) and decreased viability (by about 35%) only in myoblasts. It should be noted that the concentration of palmitate used in our study was lower than in most previous studies showing its deleterious effects in myotubes [14, 17, 18].

Mitochondrial respiration after the treatment with palmitate was affected in different ways in myoblasts and myotubes. We observed decreased basal mitochondrial respiration, uncoupler-induced maximal respiration, spare respiratory capacity (SRC) and coupling efficiency in palmitate-treated myoblasts. A decrease in all parameters of mitochondrial respiration except the proton leak in palmitate-treated myoblasts could be explained by a reduction in mitochondrial mass as the activity of citrate synthase was significantly lower in palmitate-treated myoblasts. In myotubes, uncoupler-induced maximal respiration, SRC and the citrate synthase activity were not affected after the exposure to palmitate suggesting no change in mitochondrial respiratory capacity or mitochondrial density. Palmitate caused higher basal respiration because of an increased proton leak showing a slight uncoupling of mitochondrial respiration from oxidative phosphorylation, an effect already reported for long-chain fatty acids in skeletal muscle [39]. This effect could be, at least in part, mediated through the uncoupling protein-3 (UCP-3) which is specifically expressed in differentiated muscle cells [40] since we did not observe an increase in proton leak in undifferentiated myoblasts. Although the coupling efficiency, a portion of basal respiration used for ATP synthesis, was decreased after the treatment with palmitate; the rate of ATP turnover-linked respiration itself was not affected.

The presented results show that palmitate caused similar mtDNA damage in myoblasts and myotubes, which was associated with mitochondrial respiratory dysfunction and decreased viability only in myoblasts. We hypothesize that this higher resistance of myotubes to the effect of palmitate compared to myoblasts could be in part explained by their higher

spare respiratory capacity (SRC, 75% of maximal OCR in myotubes vs. 53% in myoblasts) and therefore an increased ability to face the stress caused by palmitate. SRC is critical for survival and function of cells as it reflects the maximum ability of mitochondria to maintain energy production in response to an increase in energy demand, like in acute or chronic stress [41, 42].

Mitochondria-targeted antioxidants MitoQ and MitoTEMPOL used in this study have been previously shown to protect pancreatic β-cells against oxidative stress under glucolipotoxic conditions [24], inhibit peroxide-induced mitochondrial oxidative damage in endothelial cells [23] or prevent oxidative damage of mtDNA in myoblasts [27]. In our study, MitoTEMPOL prevented palmitate-induced mtDNA damage in C2C12 myoblasts and decreased production of hydrogen peroxide in non-treated cells confirming its antioxidant potential. In palmitate-treated myoblasts there was a trend towards a reduction of hydrogen peroxide emission but the difference did not reach statistical significance ($p=0.06$). However, we did not observe a protective effect of MitoTEMPOL against palmitate-induced cell death or mitochondrial respiratory dysfunction. MitoQ had no significant effect on palmitate-induced mtDNA damage or decreased viability of myoblasts and caused even increased hydrogen peroxide production. Pro-oxidant properties of a higher dose of MitoQ (1 μM) have been reported in certain conditions in isolated mitochondria [43, 44] while simultaneously noting its antioxidant potential. We tested even lower concentrations of antioxidants in toxicity assays, but they were also not able to prevent palmitate-induced cell death of myoblasts (Fig.4). When comparing with previous studies showing protective effects of antioxidants it should be taken into consideration that the effects will most probably depend on cell type, number of mitochondria in cells or the degree of reliance on oxidative phosphorylation.

Furthermore we found a strong inhibition of mitochondrial respiration after the treatment with both antioxidants. The functional impact of mitochondria-targeted antioxidants on cellular bioenergetics was shown recently for micromolar concentrations of MitoQ and MitoTEMPOL in mouse kidney mesangial cells [45]. We observed an inhibition of mitochondrial respiration also for 10 μM concentration of propylTPP showing that the $TPP^+$ moiety itself could be the cause of this effect. This is again in agreement with the above mentioned work of Reily et al. [45] where the acute toxicity of TPP-conjugated compounds was tested and it was found that the $TPP^+$ moiety itself has a negative impact on cellular bioenergetics. Concentrations of targeted antioxidants should therefore be carefully selected and tested for potential bioenergetics effects and an "inactive" $TPP^+$ compound should always be included as a control.

We conclude that palmitate in physiologically relevant doses affected mainly myoblasts, which were more sensitive to its effects than differentiated myotubes, possibly due to a lower spare respiratory capacity. The results suggest that skeletal muscle progenitor cells may be the first target of the deleterious action of palmitate. Mitochondria-targeted antioxidants were unable to prevent palmitate-induced cell death in myoblasts although MitoTEMPOL prevented mtDNA damage. Both antioxidants as well as propylTPP markedly inhibited mitochondrial respiration in myoblasts. These results show that the effects of mitochondria-targeted antioxidants as well as $TPP^+$ compounds on cellular bioenergetics need to be further studied and taken into account when used in cell culture studies or for therapeutic purposes.

## Disclosure Statement

The authors confirm that there are no conflicts of interest.

## Acknowledgements

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**Article 2**

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

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ORIGINAL ARTICLE

# Protective Effect of Unsaturated Fatty Acids on Palmitic Acid-Induced Toxicity in Skeletal Muscle Cells is not Mediated by PPARδ Activation

**Jana Tumova[1] · Lucia Malisova[2] · Michal Andel[1] · Jan Trnka[1]**

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**Abstract** Unsaturated free fatty acids (FFA) are able to prevent deleterious effects of saturated FFA in skeletal muscle cells although the mechanisms involved are still not completely understood. FFA act as endogenous ligands of peroxisome proliferator-activated receptors (PPAR), transcription factors regulating the expression of genes involved in lipid metabolism. The aim of this study was to determine whether activation of PPARδ, the most common PPAR subtype in skeletal muscle, plays a role in mediating the protective effect of unsaturated FFA on saturated FFA-induced damage in skeletal muscle cells and to examine an impact on mitochondrial respiration. Mouse C2C12 myotubes were treated for 24 h with different concentrations of saturated FFA (palmitic acid), unsaturated FFA (oleic, linoleic and α-linolenic acid), and their combinations. PPARδ agonist GW501516 and antagonist GSK0660 were also used. Both mono- and polyunsaturated FFA, but not GW501516, prevented palmitic acid-induced cell death. Mono- and polyunsaturated FFA proved to be effective activators of PPARδ compared to saturated palmitic acid; however, in combination with palmitic acid their effect on PPARδ activation was blocked and stayed at the levels observed for palmitic acid alone. Unsaturated FFA at moderate physiological concentrations as well as GW501516, but not palmitic acid, mildly uncoupled mitochondrial respiration. Our results indicate that although unsaturated FFA are effective activators of PPARδ, their protective effect on palmitic acid-induced toxicity is not mediated by PPARδ activation and subsequent induction of lipid regulatory genes in skeletal muscle cells. Other mechanisms, such as mitochondrial uncoupling, may underlie their effect.

**Keywords** Saturated fatty acid · Unsaturated fatty acids · Skeletal muscle cells · PPARδ · PPARδ agonist · Mitochondrial respiration · Mitochondrial uncoupling

**Abbreviations**

| | |
|---|---|
| FFA | Free fatty acids |
| PPAR | Peroxisome proliferator-activated receptors |
| PDK4 | Pyruvate dehydrogenase kinase 4 |
| UCP3 | Uncoupling protein 3 |
| CPT1 | Carnitine palmitoyltransferase 1 |

## Introduction

Saturated and unsaturated free fatty acids (FFA) differ significantly in their effects in skeletal muscle cells. Long-chain saturated FFA have been shown to induce insulin resistance (IR), inflammation, reactive oxygen species (ROS) generation, and mitochondrial damage [1–3]. These conditions are detrimental for cellular function and may eventually lead to cell death. Apoptosis has been shown to occur in skeletal muscle cells exposed to saturated palmitic acid [4, 5]. Although various mechanisms may be involved, changes in mitochondrial respiration seem to play a central role in FFA-induced cellular damage (reviewed

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in [6]). Unsaturated FFA, on the other hand, do not cause these effects and even protect muscle cells against saturated FFA-induced damage [2, 7–9] but the mechanisms involved are poorly understood. Proposed mechanisms of protective effect of unsaturated FFA include the ability to increase intracellular fatty acids disposal by promoting their mitochondrial oxidation and storage in triacylglycerols [2, 9]. Activation of these pathways decreases the level of free intracellular fatty acids available for metabolic conversions to active lipid intermediates, such as diacylglycerol (DAG) and ceramide and other pathways leading to cellular damage. DAG and/or ceramide have been implicated in the development of IR and other adverse effects in skeletal muscle/cells [8, 10, 11]. Increasing β-oxidation by genetic manipulations has been shown to protect muscle cells against palmitic acid-induced IR and apoptosis by decreasing the content of DAG and ceramide [4] but also without changes in intracellular lipid content [12].

Considering the beneficial effects of unsaturated FFA, it should be taken into account that these substances and their metabolites are natural ligands of peroxisome proliferator-activated receptors (PPAR) [13]. These nuclear receptors function as ligand-activated transcription factors that regulate lipid and energy metabolism by transcriptional activation of target genes. Fatty acids can, therefore, modulate their own metabolism by interacting with PPAR. Three isoforms of PPAR with tissue-specific expressions and functions were identified—PPARα, β/δ, and γ. In skeletal muscle, PPARδ (sometimes also referred to as PPARβ) are more abundant than either PPARα or PPARγ [14, 15]. PPARδ in skeletal muscle are known to play a critical role in the coordination of metabolic adaptations in response to fasting and endurance exercise (reviewed in [16, 17]), however, their role in conditions of lipid overload is not so well defined.

The effects of PPARδ activation have been broadly studied by using PPARδ selective synthetic agonists. PPARδ activation has been shown to induce expression of genes involved in preferential lipid utilization, fatty acid oxidation, and energy expenditure [18, 19] leading to an increased fatty acid uptake and oxidation, as demonstrated in cultured muscle cells and isolated skeletal muscle [15, 19, 20]. In contrast, activation of PPARα and PPARγ showed very little or no impact on the expression of the above-mentioned genes or fatty acid oxidation [19]. PPARδ activation also ameliorates lipid accumulation and development of IR in skeletal muscle cells exposed to palmitic acid and in skeletal muscle of high fat-fed mice [19, 21]. PPARδ-induced increase in fatty acid catabolism is considered the principal agent in its ability to prevent metabolic abnormalities in these models. Our understanding of the role and effects of natural PPARδ agonists, as opposed to synthetic agonists, is still incomplete.

Given that unsaturated FFA and their metabolites are natural ligands of PPARδ [13], and given the documented beneficial effects of PPARδ activation by synthetic agonist in skeletal muscle/cells exposed to lipid overload [19, 21], we hypothesized that the protective effects of unsaturated FFA may be mediated by PPARδ activation and subsequent induction of lipid regulatory genes. In the present study, we used C2C12 myotubes as an *in vitro* model of skeletal muscle and compared the effect of long-chain saturated FFA (palmitic acid), mono- and polyunsaturated FFA (oleic, linoleic and α-linolenic acid) and their combinations on the activation of PPARδ (expression of PPARδ and candidate PPARδ target genes). We examined also the impact of different FFA and of PPARδ activation on mitochondrial respiration, which plays an important role in FFA-induced cellular damage and has been proposed to be regulated by PPARδ [16].

## Methods

### Materials

Fatty acids and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma-Aldrich. PPARδ agonist GW501516 was from Enzo Life Sciences and PPARδ antagonist GSK0660 from Tocris Bioscience. Reagents for reverse transcription and real-time PCR were obtained from Life Technologies.

### Preparation of Stock Solutions of Fatty Acids

Fatty acids were conjugated with fatty acid-free bovine serum albumin (BSA) using a modified method described by Cousin [22]. Briefly, palmitic acid and unsaturated fatty acids were dissolved in 0.1 M sodium hydroxide by mixing and heating to 70 °C and 60 °C, respectively, to reach a final concentration of 100 mM. These solutions were then mixed with 15 % fatty acid-free BSA in Dulbecco's phosphate-buffered saline (DPBS) at 50 °C for 30 min, yielding a final stock solution of 5 mM. These steps were done under an argon atmosphere. The fatty acid:BSA molar ratio was 2:1. A control BSA solution was prepared by mixing 0.1 M sodium hydroxide with 15 % fatty acid-free BSA. The pH of all solutions was adjusted to 7.4. After sterilization by filtration the actual concentrations of fatty acids were determined using a non-esterified fatty acids kit (Randox Laboratories, UK). Stock solutions of fatty acids were kept under an argon atmosphere in −80 °C.

**Table 1** Types of FFA used in experiments

| Trivial name | Formula | Abbreviation |
|---|---|---|
| Palmitic acid | 16:0 | PAM |
| Oleic acid | 18:1(n-9) | OLA |
| Linoleic acid | 18:2(n-6) | LNA |
| α-Linolenic acid | 18:3(n-3) | ALA |

### Cell Culture and Treatment

Mouse C2C12 myoblasts (ECACC, UK) were grown in DMEM (1 g/L glucose, Life Technologies) supplemented with 10 % fetal bovine serum (FBS, Life Technologies), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich) at 37 °C in an atmosphere of 95 % humidity and 5 % $CO_2$. For all experiments, cells were seeded into experimental plates, allowed to reach confluence and differentiated to myotubes by lowering the serum concentration to 2 %. After 7 days, when full differentiation was observed, myotubes were used for experiments. Myotubes were incubated for 24 h in DMEM supplemented with 1 % FBS containing different concentrations (100, 300, or 600 μM) of FFA conjugated to BSA (Table 1), corresponding concentrations of BSA only (vehicle, C-BSA), 1 μM PPARδ agonist GW501516 (GW), 1 μM PPARδ antagonist GSK0660 (GSK), or no treatment (C).

The amount of FBS in media was decreased to 1 % for experiments to decrease the amount of serum-derived fatty acids. FBS supplementation could not be completely avoided as it caused decreased cell viability (data not shown).

### Cell Viability Assay

Cell viability was assessed using the MTS assay (Promega). The incubation medium with tested compounds was washed away and replaced by medium with the MTS reagent at the end of exposure and cells were incubated for 90 min at 37 °C. The absorbance of formazan was measured at 490 nm in a microplate reader. Data are displayed as a percentage of cells with no additions.

### Analysis of mRNA Expressions by RT-PCR

The mRNA levels of selected genes were assessed by RT-PCR. Cells were differentiated in 6-well plates and after 24 h of incubation, cells were lysed in RLT buffer and total RNA was isolated using the RNeasy Mini Kit (Qiagen). RNA concentration was measured by Nanodrop 1000 (Thermo Fisher Scientific). Genomic DNA was removed by DNAse I treatment, and cDNA was obtained by reverse transcription of 1 μg of total RNA using a high capacity cDNA reverse transcription kit. The cDNA equivalent of 5 ng or 25 ng of RNA was used for RT-PCR reactions using Universal Master Mix II and gene expression TaqMan assays (Applied Biosystems) for pyruvate dehydrogenase kinase 4 (PDK4, assay ID: Mm01166879_m1), uncoupling protein 3 (UCP3, Mm00494077_m1), muscle isoform of carnitine palmitoyltransferase 1 (CPT1, Mm00487200_m1), and PPAR$\delta$ (Mm00803184_m1). All samples were prepared in technical duplicates and were run on a 7500 Fast ABI Prism Instrument (Applied Biosystem). The mRNA expression of each gene was normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Mm99999915_g1) and fold change of expression was calculated using the ΔΔCt method.

### Analysis of Mitochondrial Respiration

Oxygen consumption rates (OCR) were measured using the XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Myoblasts were differentiated into myotubes in 24-well assay plates (Seahorse Bioscience) as described above. After 24 h incubation, the culture medium was replaced with the assay medium (XF Assay Modified DMEM supplemented with 4 mM L-glutamine, 1 mM pyruvate, and 5.5 mM glucose) and the mitochondrial stress test was performed to assess the bioenergetic profile of the myotubes [23]. OCR measurements were obtained before and after sequential additions of the ATPase inhibitor oligomycin (1 μM), inner membrane uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 1.5 μM), and inhibitors of complex I and III rotenone (1 μM) and antimycin A (1 μM). Protein content of the wells was determined by a bicinchoninic acid kit (Sigma). Data are expressed as pmol $O_2$/min/μg protein. Parameters of mitochondrial respiration were calculated as described previously [3].

### Statistical Analysis

Results are expressed as mean ± SEM of at least three independent experiments. Significant differences between treatments were assessed by one-way ANOVA followed by Dunnett post hoc comparison or *t* test, as appropriate. One-way ANOVA was performed for each group of treatments shown in individual figures with the exception of Figs. 3b and 4, which constituted one group for the purposes of statistical analysis but are presented as separate graphs for the sake of clarity. The analyses were performed using the GraphPad Prism 6 (GraphPad Software, Inc.). Threshold of significance was defined as $p < 0.05$.

## Results

### Palmitic Acid-Induced Cytotoxicity was Prevented by all Tested Unsaturated FFA

As saturated FFA, unlike unsaturated ones, are expected to have detrimental effects in skeletal muscle cells, we first evaluated the cytotoxic effect of FFA selected for our experiments within a concentration range of 100–1000 μM using the MTS assay. As expected, only palmitic acid was found to be significantly cytotoxic for myotubes after a 24 h treatment and decreased their viability in a dose-dependent manner when compared to corresponding BSA controls (~10, 25, and 35 % decrease for 300, 600, and 1000 μM PAM) (Fig. 1a). Substitution of a half dose of palmitic acid with mono- or polyunsaturated FFA prevented its cytotoxic effect, and this effect was observed even for very high total concentrations of FFA (1000 μM) (Fig. 1b).

### Changes in Expression of PPARδ Target Genes and PPARδ After Treatment with FFA, GW501516, and GSK0660

To determine the involvement of PPARδ in the protective effect of unsaturated FFA, we examined their activation via changes in mRNA expression of PPARδ target genes [15, 19] that encode for proteins involved in fatty acid oxidation (CPT1, key regulatory enzyme of mitochondrial fatty acid oxidation), preferential fatty acids utilization (PDK4, inhibits pyruvate dehydrogenase), and energy/lipid metabolism (UCP3). To verify that these genes are regulated by PPARδ in our model, cells were treated with GW501516, a highly selective PPARδ agonist [24], which markedly induced their expression (approximately fivefold increase for CPT1 and sixfold increase for PDK4 and UCP3 *vs*. control cells, Fig. 2). In contrast, PPARδ antagonist GSK0660 reduced expression of CPT1 and UCP3 below the basal levels (by approximately 20 % for CPT1 and 60 % for UCP3) but did not change expression of PDK4 (Fig. 2). A corresponding concentration of drug diluent (0.005 % DMSO) had no effect on gene expressions when compared to non-treated controls (data not shown).

The effects of various FFA on the expression of PPARδ target genes were tested for total 100, 300, and 600 μM concentrations of FFA, which we believe reflect low physiological, moderately increased, and highly increased levels of FFA in plasma, as reported in rodents *in vivo* [25, 26]. Monounsaturated FFA (OLA), as well as both polyunsaturated FFA (LNA and ALA), significantly induced PPARδ target genes expressions in a dose-dependent manner (Fig. 3). Maximal induction was achieved by 300 μM fatty acids and higher doses did not further increase gene expression,

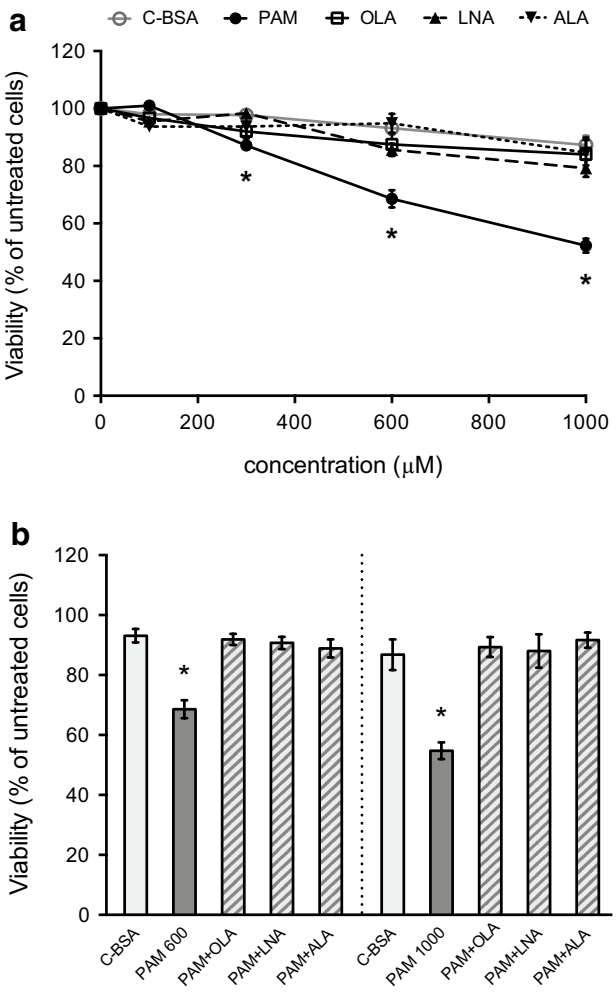


**Fig. 1** Viability of myotubes assessed by the MTS assay. **a** Myotubes were incubated with various FFA in concentrations varying from 100 to 1000 μM or corresponding concentrations of BSA (C-BSA) for 24 h. **b** Myotubes were incubated with 600, 1000 μM palmitic acid (PAM), or with combination of PAM and unsaturated FFA (OLA, LNA, ALA) in a ratio 1:1 for 24 h. Data are displayed as a percentage of untreated cells and presented as mean ± SEM ($n = 4$). *$p < 0.05$ compared to BSA control (C-BSA)

except for UCP3. Saturated FFA (PAM) induced PPARδ target gene expression only at 300 μM (CPT1 and UCP3) and 600 μM concentrations (all genes), but this effect was significantly lower than observed for any of unsaturated FFA. Interestingly, substitution of a half dose of PAM with unsaturated FFA did not lead to expected increase in gene expression over the levels observed for PAM alone. This effect was observed for all tested concentrations. On the other hand, the combination of PAM with GW501516 led to a marked induction of gene expression compared to PAM alone (Fig. 3). To confirm that the effects of FFA on gene expression are really mediated by PPARδ, the antagonist GSK0660 was added to some of the treatments (300 μM

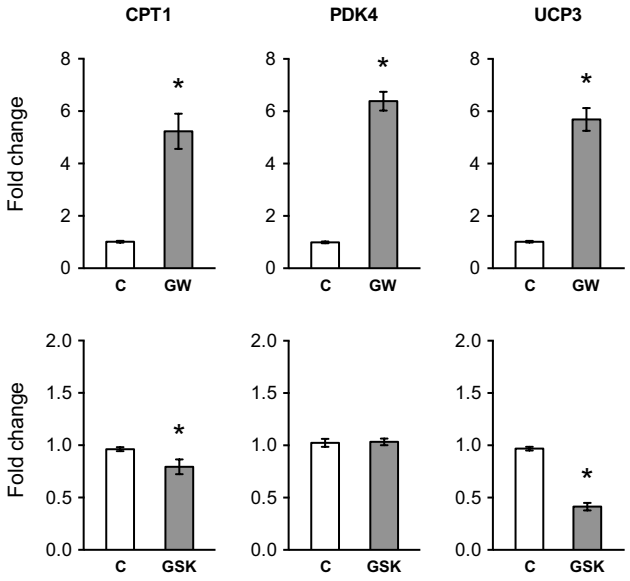


**Fig. 2** The mRNA expression of PPARδ target genes CPT1, PDK4, and UCP3. Myotubes were treated with 1 μM PPARδ agonist GW501516 or 1 μM PPARδ antagonist GSK0660 for 24 h. The mRNA expression of target genes relative to the internal control GAPDH was determined by RT-PCR analysis and fold change of expression was calculated using the ΔΔCt method. Results are presented as mean ± SEM ($n = 3$). *$p < 0.05$ compared to control (C)

PAM and 300 μM OLA). The addition of GSK0660 completely prevented PAM or OLA-induced increase in mRNA expression of all assessed genes indicating that this increase was mediated through an activation of PPARδ (results shown separately in Fig. 4 with C-BSA, PAM and OLA representing the experimental groups from Fig. 3b).

To explore whether changes in PPARδ activity after FFA treatments are associated with differences in PPARδ expression, we also measured its mRNA level. However, PPARδ mRNA level was not significantly altered in any of the treatments (FFA, GW501516, or GSK), with the exception of a few cases where only a very low increase was observed (approx. 1.5-fold, Suppl. Fig. 1).

## Palmitic Acid-Induced Cytotoxicity Was Not Prevented by GW501516 or Other Compounds Modulating Fatty Acid Oxidation

PPARδ activation by GW501516 has been reported to prevent palmitic acid-induced cellular disturbances, such as IR or inflammation [19, 21]. Therefore, we were interested in whether GW501516 could prevent palmitic acid-induced cell death in C2C12 myotubes. We found no difference in the viability of cells treated with 600 μM PAM and cells co-incubated with PAM and GW501516 (Fig. 5). To test whether other alterations of fatty acid oxidation may affect PAM cytotoxicity we co-incubated cells for 24 h with PAM + 0.5 mM carnitine to enhance FFA oxidation or PAM + 40 μM etomoxir to block FFA entry into mitochondria and their oxidation. No difference in the viability of cells was observed for co-incubated cells compared to PAM-treated cells (Fig. 5). The 1 μM GW501516 alone had no significant effect on cell viability after 24 h of treatment (104.7 ± 3.1 % viable cells, as a percentage of untreated cells). No significant effect was observed also for vehicle (0.005 % DMSO) (101.3 ± 1.9 %), 0.5 mM carnitine (95.9 ± 3.7 %), or 40 μM etomoxir (96.3 ± 3.1 %).

## Effects of FFA and GW501516 on Mitochondrial Respiration of Myotubes

FFA, mainly saturated ones, have been shown to affect some features of mitochondrial respiration [8, 27]. PPARδ have been suggested to play a role in the regulation of mitochondrial function [16], but their direct effects on mitochondrial respiration in muscle cells are still unknown. Therefore, we assessed the effect of FFA (moderate or high doses) and PPARδ agonist on basic parameters of mitochondrial respiration in myotubes. FFA at 300 μM total concentration had little impact on mitochondrial respiration. Basal and maximal respiration (Suppl. Fig. 2a) as well as ATP turnover-linked respiration were not affected. Significant difference was found only in proton leak-driven mitochondrial respiration. Increased proton leak and, therefore, decreased coupling efficiency was observed for unsaturated FFA and for mixtures of PAM and unsaturated FFA when compared to non-treated cells, although the difference was not statistically significant for all treatments (Fig. 6a). An uncoupling effect on mitochondrial respiration, without affecting ATP turnover-linked respiration, was found also for the agonist GW501516 (Fig. 6c).

FFA at 600 μM total concentration had, as expected, more pronounced effects on mitochondrial respiration. There was no statistically significant difference in proton leak but all treatments, including BSA, except for OLA and its combination with PAM, caused a statistically significant decrease in ATP turnover-linked respiration (Fig. 6b). Maximal respiration was not significantly different between treatments, although there was a tendency to decrease for palmitic acid and the combination of palmitic acid with GW501516 (Suppl. Figure 2b).

Corresponding concentration of drug diluent (0.005 % DMSO) had no effect on mitochondrial respiration when compared to non-treated controls (data not shown).

## Discussion

Unsaturated FFA, mainly monounsaturated oleic acid, have been shown to protect skeletal muscle cells against the

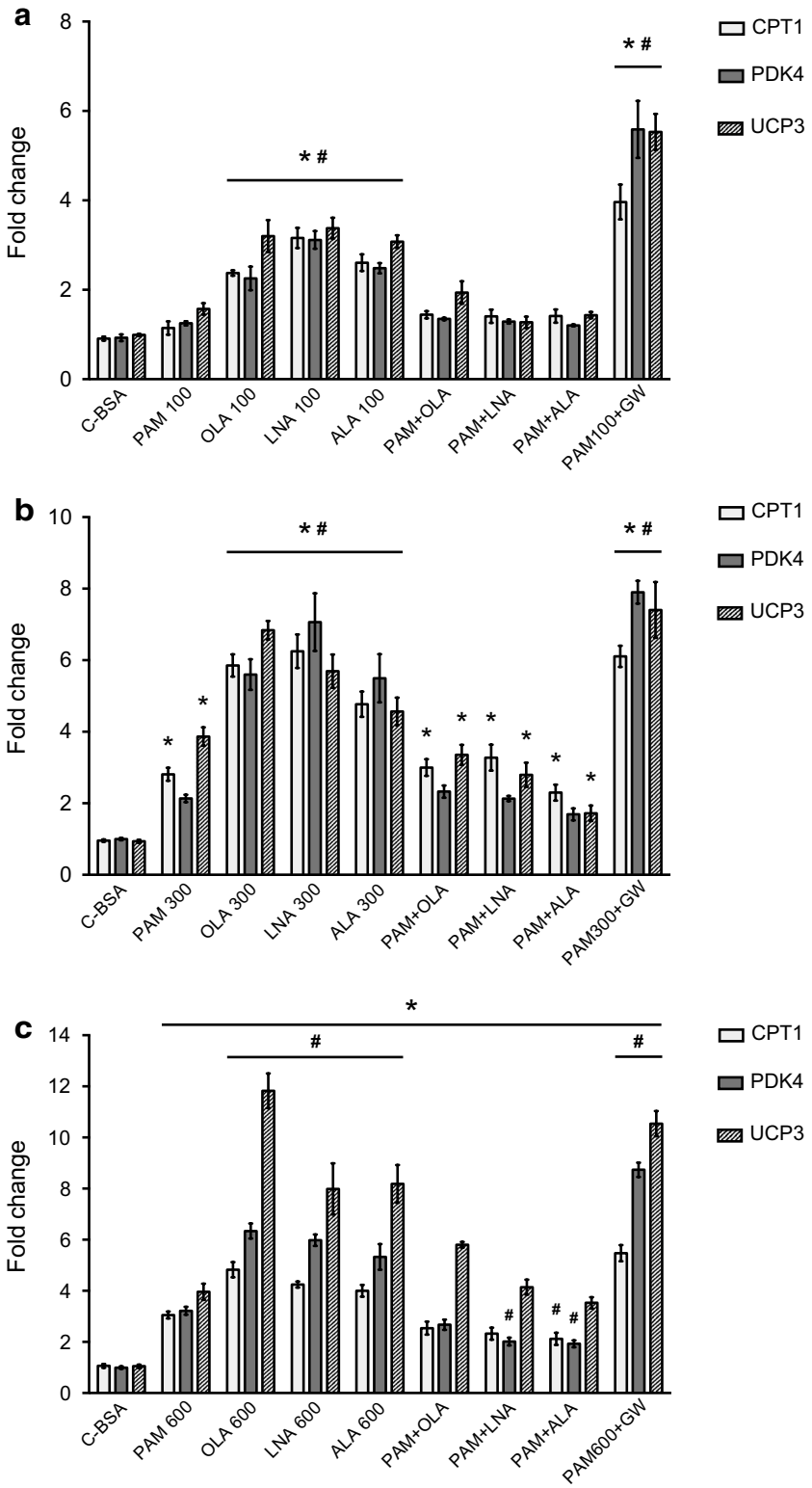


**Fig. 3** The mRNA expression of PPARδ target genes CPT1, PDK4, and UCP3. Myotubes were treated with 100 μM (**a**), 300 μM (**b**), and 600 μM (**c**) total concentration of palmitic acid (PAM), unsaturated FFA (OLA, LNA, ALA) or with combinations: PAM + unsaturated FFA in a ratio 1:1 or PAM + 1 μM GW501516 for 24 h. The mRNA expression of target genes relative to the internal control GAPDH was determined by RT-PCR analysis and fold change of expression was calculated using the ΔΔCt method. Results are presented as mean ± SEM ($n = 3$). $^{*}p < 0.05$ compared to BSA control (C-BSA), $^{\#}p < 0.05$ compared to PAM

lipotoxic action of palmitic acid [2, 4, 8]. In our study, we observed the protective effect against palmitic acid-induced cytotoxicity not only for oleic acid, but also for polyunsaturated fatty acids of the n-6 (linoleic) and n-3 (α-linolenic) series. This effect was observed for 600 μM and also for 1000 μM total concentration of FFA, which could be considered very high since the highest reported levels in plasma of rodents after a high-fat diet or fasting did not

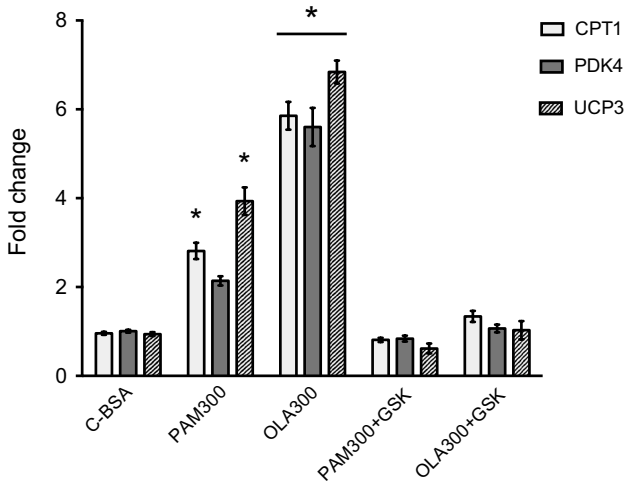


**Fig. 4** The mRNA expression of PPARδ target genes CPT1, PDK4, and UCP3. Myotubes were treated with 300 μM palmitic (PAM) or oleic acid (OLA) for 24 h in the presence or absence of PPARδ antagonist GSK0660. The mRNA expression of target genes relative to the internal control GAPDH was determined by RT-PCR analysis and fold change of expression was calculated using the ΔΔCt method. Results are presented as mean ± SEM ($n = 3$). *$p < 0.05$ compared to BSA control (C-BSA)

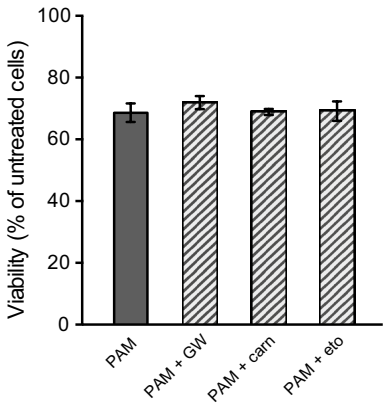


**Fig. 5** Viability of myotubes after the treatment with 600 μM palmitic acid (PAM) in the presence or absence of 1 μM PPARδ agonist GW501516, 0.5 mM carnitine (carn), or 40 μM etomoxir (eto) for 24 h. Viability was assessed by the MTS assay. Data are displayed as a percentage of untreated cells and presented as mean ± SEM ($n = 4$)

exceed 700 μM [25, 26]. Unsaturated FFA must, therefore, initiate an efficient mechanism to protect cells against FFA load. The aim of the present study was to determine the role of PPARδ, transcriptional regulators of genes involved in lipid metabolism, in the protective effect of unsaturated FFA in skeletal muscle cells.

We assessed mRNA expression of CPT1, PDK4, and UCP3 genes, which were all similarly upregulated by PPARδ agonist GW501516 confirming that they are targets of PPARδ regulation. Basal level of CPT1 and UCP3, but not PDK4, mRNA expression was downregulated by PPARδ antagonist GSK0660. The lack of repression for PDK4 by GSK0660 has been previously reported and can be explained by low basal levels of PDK4 expression, which is already maximally repressed [28]. Unsaturated FFA increased mRNA expression of PPARδ target genes to the same degree as PPARδ selective agonist and, therefore, proved to be effective PPARδ activators in C2C12 myotubes. Although primarily polyunsaturated FFA and their metabolites have been proposed as effective agonists of PPARδ [13], in our experiments monounsaturated oleic acid exhibited similar effects. Saturated palmitic acid acted as a weak activator of PPARδ in myotubes as was previously suggested [13]. Contrary to our expectations, when unsaturated FFA were mixed with palmitic acid, the activation of PPARδ was repressed, i.e., stayed at the levels observed for saturated FFA alone. This effect was observed for all assessed genes and across all tested concentrations. It is possible that it may be a result of an unknown interaction of palmitic acid and unsaturated FFA since the combination of palmitic acid with GW501516 markedly increased PPARδ activity.

Ligand binding seems to play a more important role in regulating receptor activity than changes in receptor expression, as PPARδ mRNA levels were unchanged after most FFA or agonist treatments. The small increase after a few FFA treatments, which did not correspond with changes in expression of target genes, does not seem to be biologically relevant. Another possibility is that PPARδ expression is regulated more dynamically, and we were not able to register changes after 24 h of incubation, as one study reported slightly upregulated (<twofold) mRNA expression of PPARδ by oleic/linoleic acid mixture in cultured myotubes after 3 h and 12 h of incubation but not in other time points [26].

Altogether, our results from gene expression assays indicate that PPARδ activation does not seem to be involved in the protective effect of unsaturated FFA. Moreover, manipulations of fatty acid oxidation by PPARδ agonist, carnitine, or etomoxir had no impact on palmitic acid-induced cytotoxicity, suggesting that its effect is not mediated by a lack of PPARδ activation or insufficient fatty acid oxidation. Indeed, a recent study showed that oleic acid prevents palmitic acid-induced ER stress and inflammation through an AMP-activated protein kinase (AMPK)-dependent mechanism, without an involvement of PPAR or increased fatty acid oxidation [29]. Interestingly, some effects of PPARδ agonist GW501516 in skeletal muscle cells also appear to be mediated by PPARδ-independent and AMPK-dependent mechanisms [20]. The protective effect of unsaturated FFA may also involve the channeling of palmitic acid into triacylglycerols, rather than an activation of β-oxidation; this mechanism has been reported for oleic and arachidonic acid [2, 4, 30].

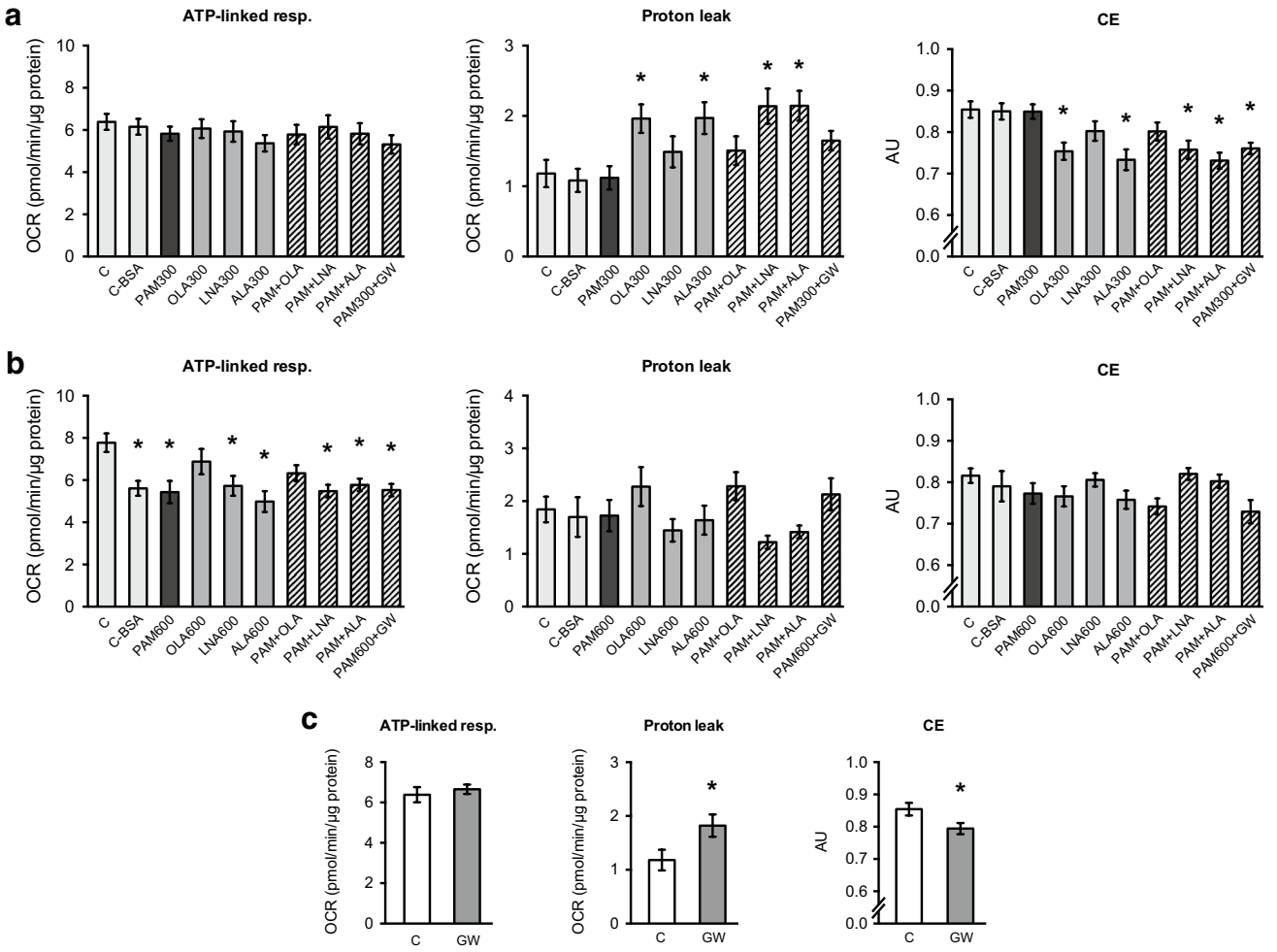


**Fig. 6** Mitochondrial respiration (oxygen consumption rates, OCR) in myotubes measured under different respiratory conditions. Myotubes were treated with 300 µM (**a**) or 600 µM (**b**) total concentration of palmitic acid (PAM), unsaturated FFA (OLA, LNA, ALA) and combinations: PAM + unsaturated FFA in ratio 1:1 or PAM + 1 µM GW501516 for 24 h. **c** Myotubes were treated with 1 µM GW501516 for 24 h. ATP turnover-linked respiration, proton leak and coupling efficiency (CE, the ratio of ATP turnover-linked and basal respiration) were assessed by a stress test as described in "Methods" section. Results are presented as mean ± SEM ($n \geq 3$). *$p < 0.05$ compared to control (C)

Saturated FFA, but not unsaturated FFA, have been shown to affect mitochondrial respiration in skeletal muscle cells [8, 27]. However, the only effect observed in our mitochondrial respiration analysis at 300 µM FFA concentration (corresponding to moderately elevated levels) was mitochondrial uncoupling observed for unsaturated FFA as well as for their combinations with palmitic acid but not for palmitic acid alone. The protonophoric action of long-chain FFA has been well described; however, the specificity of various fatty acids is less clear [31]. We suggest that the beneficial effects of unsaturated FFA, at least at this dose, might be connected with their ability to partially uncouple mitochondria as this will increase energy expenditure and increase fatty acids removal [32]. Mild uncoupling was observed also for the PPARδ agonist and could also explain some of its beneficial effects during situations of lipid overload. Our assumption is in agreement with the above-mentioned role of AMPK in the effects of agonist and unsaturated FFA as uncoupling can result in an increased AMP:ATP ratio in the cell and activation of AMPK [33].

At the FFA concentration of 600 µM (corresponding to highly elevated levels) uncoupling was no longer evident, but there was a decreased basal and ATP turnover-linked respiration for all treatments except for oleic acid and the combination of oleic and palmitic acid. However, the fall in the ATP turnover-linked respiration was not so large as to affect viability of cells exposed to unsaturated FFA or BSA, as only palmitic acid was cytotoxic at this dose. Although all unsaturated FFA were able to prevent palmitic acid-induced cell death, only oleic acid prevented the drop in ATP-linked respiration. The mechanism of this effect is unclear and needs to be further elucidated. Time course

studies of respiration would be helpful as prolonged exposition to FFA may result in an exhaustion of compensatory mechanisms, such as mitochondrial uncoupling, and to mitochondrial and cellular damage [34].

In summary, results from the present study demonstrate that palmitic acid is highly cytotoxic for differentiated skeletal muscle cells when present alone, but this effect is abolished by the presence of unsaturated FFA. Although FFA are important regulators of PPARδ, PPARδ activation and subsequent induction of target lipid regulatory genes do not seem to be involved in the protective effect of unsaturated FFA against palmitic acid-induced lipotoxicity. Other mechanisms, such as the increase in energy expenditure by mild uncoupling, AMPK activation, or channeling of FFA into triacylglycerols may explain the beneficial effects of unsaturated FFA and these mechanisms need to be further clarified.

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**Compliance with Ethical Standards**

**Conflict of interest** The authors confirm that there are no conflicts of interest.

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**Article 3**

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

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

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ORIGINAL ARTICLE

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

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**BACKGROUND/OBJECTIVES:** Vegans have a lower incidence of insulin resistance (IR)-associated diseases and a higher insulin sensitivity (IS) compared with omnivores. The aim of this study was to examine whether the higher IS in vegans relates to markers of mitochondrial biogenesis and to intramyocellular lipid (IMCL) content.

**SUBJECTS/METHODS:** Eleven vegans and 10 matched (race, age, sex, body mass index, physical activity and energy intake) omnivorous controls were enrolled in a case–control study. Anthropometry, bioimpedance (BIA), ultrasound measurement of visceral and subcutaneous fat layer, parameters of glucose and lipid homeostasis, hyperinsulinemic euglycemic clamp and muscle biopsies were performed. Citrate synthase (CS) activity, mitochondrial DNA (mtDNA) and IMCL content were assessed in skeletal muscle samples.

**RESULTS:** Both groups were comparable in anthropometric and BIA parameters, physical activity and protein–energy intake. Vegans had significantly higher glucose disposal (*M*-value, vegans 8.11 ± 1.51 vs controls 6.31 ± 1.57 mg/kg/min, 95% confidence interval: 0.402 to 3.212, $P = 0.014$), slightly lower IMCL content (vegans 13.91 (7.8 to 44.0) vs controls 17.36 (12.4 to 78.5) mg/g of muscle, 95% confidence interval: − 7.594 to 24.550, $P = 0.193$) and slightly higher relative muscle mtDNA amount (vegans 1.36 ± 0.31 vs controls 1.13 ± 0.36, 95% confidence interval: − 0.078 to 0.537, $P = 0.135$). No significant differences were found in CS activity (vegans 18.43 ± 5.05 vs controls 18.16 ± 5.41 μmol/g/min, 95% confidence interval: − 4.503 to 5.050, $P = 0.906$).

**CONCLUSIONS:** Vegans have a higher IS, but comparable mitochondrial density and IMCL content with omnivores. This suggests that a decrease in whole-body glucose disposal may precede muscle lipid accumulation and mitochondrial dysfunction in IR development.

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**Keywords:** vegan; insulin resistance; IMCL; mitochondrial density

## INTRODUCTION

Insulin resistance (IR)-associated diseases such as obesity and type 2 diabetes (T2DM) rank among the major risk factors of atherosclerosis, and its complications contribute greatly to cardiovascular mortality in developed countries. Composition of diet is considered to be one of the causes of IR. People consuming a strict plant-based diet (vegans) have more favorable parameters of glucose tolerance, lipid profile and lower IR compared with their counterparts without food restriction.[1–3] The prevalence of T2DM is lower in vegan populations as well.[4,5] In line with these findings, the use of vegetarian diets in T2DM patients has been shown to improve diabetes compensation, lipid profile and to lower IR.[6,7]

Development of IR is closely related to skeletal muscle metabolic function, as the skeletal muscle is normally responsible for up to 85% of insulin-stimulated glucose uptake.[8] Insulin sensitivity (IS) of the muscle is also influenced by circulating the plasma free fatty acids (FFAs) and the related accumulation of intramyocellular lipids (IMCLs), and by mitochondrial function. IMCL likely interferes with intracellular insulin signaling, as the IMCL content has been shown to be positively correlated with IR.[9,10] However, higher IMCL content has also been found among endurance athletes[11] who are generally insulin sensitive, which further complicates the causal model.

Previous studies have shown that IRs and IMCLs are correlated with reduced amounts of mitochondria and a related loss of oxidative capacity. Indeed, obese and T2DM patients have lower mitochondrial content[12,13] and lower skeletal muscle oxidative capacity; in addition, their mitochondrial cross-sectional area is reduced ~30% (Kelly *et al.*[14]) and the electron transport chain activity is reduced as well.[13] Nevertheless, the relationship between IR, IMCL and mitochondrial dysfunction is complex, causality is unclear and results from available studies are often controversial.[15,16]

It has already been shown that vegans have higher IS and lower IMCL content compared with omnivores.[2] There are currently no published data addressing mitochondrial function in vegans and a possible relationship between reported higher IS, lower IMCL and muscle mitochondrial function. The aim of this study was to assess whether higher IS and lower IMCL in vegans were associated with altered muscle mitochondrial density.

## SUBJECTS AND METHODS

### Study sample

A total of 11 Caucasian vegan subjects (6 male and 5 female) comprised the first study group. These subjects had followed a strict vegan diet

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(no meat, no dairy products and no eggs) for more than 3 years before enrolling to the study (mean time on vegan diet was 8.05 years). The control group contained 10 subjects (6 male and 4 female) with no food restrictions, consuming meat and other animal products on a daily basis. The control group was selected to match the vegan group in race, gender, age, body mass index, anthropometry, bioimpedance (BIA) analysis, physical activity and energy intake.

Exclusion criteria for recruitment included the following: age under 18 years, obesity, any chronic disease related to energy metabolism (particularly diabetes, thyropathy, hypertension, dyslipidemia, atherosclerosis and so on), any chronic medication (including hormonal contraception), smoking (even in past medical history) and regular alcohol consumption. Subjects with close relatives suffering from T2DM and those contraindicated for muscle biopsy were also excluded.

The research protocol was approved by the Ethics Committee of the Third Faculty of Medicine of the Charles University, Prague, and the Ethics Committee of Faculty Hospital Kralovske Vinohrady in accordance with the Declaration of Helsinki. Each participant gave an informed consent before being enrolled in study.

### Anthropometry and clinical examination

Each subject underwent a basic anthropometric examination (height (m), weight (kg), body mass index (kg/m$^2$), waist circumference (cm) and waist–hip ratio). The arm, thigh and calf circumference and skinfold thickness were measured to calculate total muscle mass.[17] Body composition was measured using BIA analysis (Nutriguard-M, Data Input GmbH, Frankfurt, Germany). The amount of visceral fat was assessed using ultrasonography (Philips iU22, Best, Netherlands) as the omental fat layer thickness (distance between the abdominal fascia and aorta at the umbilical level). Each of these measurements was performed three times and the mean value was recorded.

### Dietary assessment

Each participant filled in a prospective questionnaire with dietary data from 3 days (2 working days and 1 weekend day). The NutriDan program was used for dietary intake calculations. As nutritional data for certain vegan products were not available in the database, vegans were asked to collect packages of these products and the manufacturer's nutritional values were used for calculations. Carbohydrate, lipid and protein intakes were calculated separately.

### Physical activity assessment

Physical activity was assessed using the Baecke questionnaire for habitual physical activity,[18] the scores from which correlate well with maximum oxygen consumption ($VO_2$max).[19] Physical activity at work, leisure time and sport activities were assessed separately.

### Laboratory analysis

Peripheral venous blood sample was drawn from each subject after 12 h of fasting. Parameters of glucose homeostasis were assessed as follows: plasma glucose using the hexokinase reaction kit (KONELAB, Dreieich, Germany); C-peptide using solid-phase competitive chemiluminescent enzyme immunoassay (Immulite 2000, Los Angeles, CA, USA); HbA1c using high-pressure liquid boronate affinity chromatography (Primus Corporation, Kansas city, MO, USA); and insulin using solid-phase competitive chemiluminescent enzyme immunoassay (Immulite 2000). For the lipid profile, we measured total cholesterol and triglycerides using an enzymatic method kit (KONELAB); high-density lipoprotein–cholesterol measured using a polyethylene glycol-modified enzymatic assay kit (ROCHE, Basel, Switzerland); and low-density lipoprotein–cholesterol calculated using the standard Friedewald equation. Plasma levels of FFA were measured using the method described by Husek *et al.*[20] FFAs were extracted together with neutral lipids into isooctane and cleaned using a reverse-extraction process. FFAs obtained in this way were derivatized to methyl esters and subsequently analyzed by gas chromatography.

### Insulin sensitivity

IS was assessed in a 2-h hyperinsulinemic euglycemic clamp as described by DeFronzo *et al.*[21] After 12 h of fasting, basal biochemical tests were performed in a blood sample (plasma glucose, insulin and C-peptide) and infusions of insulin (Humulin R, Eli Lilly, Prague, Czech Republic) in a standard dose of 1 mIU/kg/min and of 15% glucose solution were started. Blood glucose was measured every 5 min using a Precision PCX glucometer (Abbott Laboratories, Wiesbaden, Germany). Three consecutive blood tests were performed for plasma insulin during the last 30 min of the clamp protocol after a steady state had been reached. C-peptide was measured at the 120th minute as a confirmation of a sufficient suppression of endogenous insulin secretion. The mean steady-state infusion rate during the clamp (six consecutive measurements) was used for calculations. IS was then expressed as the glucose disposal rate (*M*-value, mg/kg/min) after a correction for changes in the glucose pool in the extracellular fluid (space correction).

### Muscle biopsy, IMCLs and mitochondrial density in skeletal muscle

A biopsy of the vastus lateralis muscle was performed using the Bergström technique[22] under basal fasting conditions and ~200 mg of muscle was obtained. The sample was immediately microdissected under a binocular microscope, weighed, divided for respective analyses and frozen in liquid nitrogen.

Mitochondrial density was assessed using citrate synthase (CS) activity and the relative amount of mitochondrial DNA (mtDNA). CS activity was determined in muscle homogenates using the CS assay kit (Sigma, St Louis, MO, USA). Briefly, ~20 mg of muscle was homogenized in 400 μl of CelLytic MT lysis buffer (Sigma) using 2 ml Dounce homogenizer. Homogenates were centrifuged at 14 000 *g* (10 min, 4 °C) and the enzyme activity in the supernatant was assessed. CS activity was measured spectrophotometrically at 25 °C and 412 nm, and was expressed in μmol/min/g of tissue.

Relative amounts of mtDNA to nuclear DNA were determined using semiquantitative PCR to assess the amount of mtDNA per cell. Genomic and mtDNA were isolated from ~20 mg of muscle sample using the DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The tissue protocol was used, as the protocol for DNA extraction of cultured cells involves an isolation of nuclei and hence the loss of mtDNA. A 141-bp fragment of nuclear DNA and a 221-bp fragment of mtDNA were amplified from 15 ng of total DNA per tube. The nuclear DNA target primer sequences were as follows: forward primer 5′-CGAGTAAGA GACCATTGTGGCAG-3′, reverse primer 5′-GGGGCTTGTAGGCATTTGCT-3′; and for mtDNA target: forward primer 5′-TTTCATCATGCGGAGATGTTG GATGG-3′, reverse primer 5′-CCCCACAAACCCCATTACTAAACCCA-3′. The number of cycles for both fragments was determined using a cycle test to keep the amplification rate within the exponential range of the PCR. The total amount of isolated DNA and PCR products were quantified fluorometrically using a Qubit dsDNA HS Assay Kit and a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA).

IMCL content was assessed using gas chromatography to measure fatty acid content in muscle fibers. The method described by Lepage and Roy[23] with modifications by Rodrigues-Palmero *et al.*[24] was used for profiling and measuring the amount of FFA in samples. In brief, the method involves a chloroform/methanol extraction of freeze-dried muscle to isolate lipids and subsequent transesterification or esterification of FFA bound to lipids to form methyl esters, which were then analyzed using gas chromatography.

### Statistical analysis

Data were tested for normality using the Shapiro–Wilk test. For normally distributed data, samples were compared using the Student's *t*-test to test the statistical significance of differences between independent groups. The Pearson's correlation coefficient *R* was calculated to express relationships between variables. The Mann–Whitney test was used for not normally distributed data. Data are presented in text and tables as means ± s.d. with a 95% confidence interval for the difference; not normally distributed data are presented as medians and ranges. The differences at $P<0.05$ were considered statistically significant. Statistica 9.0 (StatSoft, Inc., Tulsa, OK, USA) was used to perform all statistical procedures.

## RESULTS

### Sample characteristics

Table 1 summarizes characteristics of both groups. Both groups were statistically comparable in terms of race, gender, anthropometry (body mass index and the waist–hip ratio) and BIA characteristics, that is, fat mass and fat-free mass. There was an apparent trend toward higher age in the vegan group (difference of means, 2.51 years, $P=0.084$). In addition, vegans had a trend

**Table 1.** Group characteristics for vegans and controls

| | *Vegans (n = 11)* | *Controls (n = 10)* | *95% CI for difference* | P-*value* |
|---|---|---|---|---|
| Age (years) | 29.47 (25.8–36.8) | 27.23 (25.6–28.9) | 2.24 (−0.483 to 5.881) | 0.084 |
| Height (m) | 1.75 ± 0.09 | 1.77 ± 0.07 | −0.02 (−0.088 to 0.058) | 0.665 |
| Weight (kg) | 68.63 ± 14.02 | 73.15 ± 12.82 | −4.53 (−16.83 to 7.789) | 0.451 |
| BMI (kg/m$^2$) | 22.15 ± 3.02 | 23.24 ± 2.89 | −1.09 (−3.791 to 1.620) | 0.412 |
| Waist circumference (cm) | 78.27 ± 10.91 | 82.0 ± 6.98 | −3.73 (−12.19 to 4.74) | 0.368 |
| WHR | 0.86 ± 0.10 | 0.89 ± 0.14 | −0.03 (−1.138 to 0.080) | 0.583 |
| Length of vegan diet (years) | 8.05 ± 3.83 | | | |
| *Body composition (BIA analysis, anthropometry and ultrasound fat distribution)* | | | | |
| BIA fat mass (kg) | 9.22 ± 6.03 | 11.98 ± 8.39 | −2.76 (−9.776 to 4.261) | 0.419 |
| BIA lean body mass (kg) | 57.56 ± 13.38 | 62.01 ± 11.12 | −4.46 (−16.451 to 7.529) | 0.443 |
| SM (kg) | 29.70 ± 7.60 | 27.51 ± 5.14 | 2.19 (−3.80 to 8.18) | 0.454 |
| Visceral fat—US (cm) | 4.11 ± 2.28 | 2.96 ± 1.1 | 1.15 (−0.587 to 2.875) | 0.181 |
| Subcutaneous fat—US (cm) | 1.29 (0.5–2.4) | 1.93 (0.46–4.6) | −0.64 (−0.258 to 1.779) | 0.178 |
| *Physical activity* | | | | |
| Baecke score | 9.89 ± 1.42 | 8.88 ± 1.12 | 1.01 (−0.172 to 2.180) | 0.090 |
| *Macronutrient intake* | | | | |
| Total energy (kcal) | 2097.67 ± 531.18 | 2023.00 ± 563.05 | 74.67 (−456.84 to 606.17) | 0.771 |
| Lipids (%) | 36.13 ± 7.65 | 42.31 ± 8.49 | −6.18 (−14.029 to 1.680) | 0.116 |
| Carbohydrates (%) | 48.90 ± 10.57 | 40.39 ± 7.51 | 8.51 (−0.288 to 17.320) | 0.057 |
| Proteins (g) | 74.78 ± 22.96 | 81.10 ± 19.63 | −6.32 (−26.935 to 14.291) | 0.526 |
| Proteins (%) | 14.96 ± 5.08 | 17.31 ± 5.23 | −2.35 (−7.345 to 2.655) | 0.336 |

Abbreviations: BIA, bioimpedance; BMI, body mass index; CI, confidence interval; WHR, waist–hip ratio; SM, total skeletal muscle mass calculated using arm, thigh and calf muscle circumferences; US, ultrasound.

**Table 2.** Lipid profile

| | *Vegans (n = 11)* | *Controls (n = 10)* | *95% CI for difference* | P-*value* |
|---|---|---|---|---|
| Total cholesterol (mmol/l) | 3.48 ± 0.58 | 4.20 ± 0.88 | −0.72 (−1.401 to −0.045) | 0.038 |
| HDL–cholesterol (mmol/l) | 1.38 ± 0.37 | 1.66 ± 0.40 | −0.28 (−0.631 to 0.073) | 0.114 |
| LDL–cholesterol (mmol/l) | 1.81 ± 0.64 | 2.22 ± 0.68 | −0.41 (−1.012 to 0.190) | 0.167 |
| Triacylglycerols (mmol/l) | 0.65 ± 0.26 | 0.72 ± 0.26 | −0.07 (−0.309 to 0.164) | 0.527 |
| *Plasma FFAs (μg/ml of plasma)* | | | | |
| LA (C18:2n6cc) | 40.57 ± 14.29 | 16.76 ± 8.08 | 23.81 (13.050 to 34.572) | 0.001 |
| ALA (C18:3n3) | 2.18 ± 1.14 | 0.98 ± 0.64 | 1.2 (0.342 to 2.052) | 0.009 |
| EDA (C20:2n6) | 1.67 ± 0.13 | 1.52 ± 0.10 | 0.15 (0.041 to 0.254) | 0.009 |
| DGLA (C20:3n6) | 1.39 ± 0.13 | 1.24 ± 0.17 | 0.15 (0.009 to 0.280) | 0.038 |
| Total FFA | 172.63 ± 78.43 | 119.58 ± 53.59 | 53.05 (−8.956 to 115.066) | 0.089 |
| SFA | 40.62 ± 18.97 | 39.18 ± 15.60 | 1.44 (−14.523 to 17.403) | 0.852 |
| MUFA | 85.08 ± 45.70 | 58.85 ± 30.81 | 26.23 (−9.763 to 62.218) | 0.144 |
| PUFA | 46.94 ± 15.67 | 21.85 ± 9.03 | 25.09 (13.238 to 36.931) | 0.001 |

Abbreviations: ALA, α-linolenic fatty acid; CI, confidence interval; DGLA, dihomo-γ-linolenic; EDA, eicosadienoic acid; FFA, free fatty acid; HDL, high-density lipoprotein; LA, linoleic acid; LDL, low-density lipoprotein; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

toward a higher visceral (1.15 cm, $P = 0.181$) and lower subcutaneous fat amount (−0.64 cm, $P = 0.178$), and higher physical activity (1.01 points, $P = 0.09$). Groups did not differ significantly in overall energy intake (carbohydrates, lipids and proteins).

### Plasma lipid profile

Fasting plasma lipid profiles are presented in Table 2. Vegans had lower plasma level of total cholesterol (−0.72 mmol/l, $P = 0.038$). Other parameters (plasma levels of triacylglycerols, low-density lipoprotein– and high-density lipoprotein–cholesterol) were not significantly different. Vegans had higher levels of total plasma polyunsaturated fatty acid (25.09 μg/ml, $P = 0.001$) and a clear trend toward higher total plasma FFA (53.05 μg/ml, $P = 0.089$). The results showed higher levels of omega-3 α-linolenic fatty acid (ALA; 1.20 μg/ml, $P = 0.009$) and omega-6 linoleic acid (23.81 μg/ml, $P = 0.001$), eicosadienoic acid (0.15 μg/ml, $P = 0.009$) and dihomo-γ-linolenic (0.15 μg/ml, $P = 0.038$) in vegans.

### Glucose homeostasis

Glucose homeostasis parameters are summarized in Table 3. Fasting plasma glucose was lower in vegans (−0.64 mmol/l, $P = 0.04$), whereas insulin and glycosylated hemoglobin showed no significant differences. Glucose consumption during the steady-state hyperinsulinemic clamp is shown in Figure 1. Vegans had a higher *M*-value compared with controls (1.82 mg/kg/min, $P = 0.023$), as well as *M*-values adjusted for total skeletal muscle mass (calculated from the arm, thigh and calf circumferences (1.80 mg/kg/min, $P = 0.014$)). Both groups reached comparable

**Table 3.** Hyperinsulinemic euglycemic clamp, glucose homeostasis

| | *Vegans* (n = *11)* | *Controls* (n = *10)* | *95% CI for difference* | P-*value* |
|---|---|---|---|---|
| Fasting glucose (mmol/l) | 4.82 ± 0.65 | 5.46 ± 0.69 | − 0.64 ( − 1.251 to − 0.032) | 0.040 |
| Fasting insulin (mU/l) | 1.99 (1.2-4.9) | 3.60 (1.7–8.8) | − 1.61 ( − 3.270 to − 0.096) | 0.072 |
| HbA1c (%) | 3.50 (3.1-3.9) | 3.35 (3.1–4.1) | 0.15 ( − 0.340 to 0.240) | 0.673 |
| *M*-value (mg/kg/min) | 8.18 ± 1.62 | 6.36 ± 1.74 | 1.82 (0.286 to 3.350) | 0.023 |
| *M*-value$_{corrected}$ (mg/kg/min) | 8.11 ± 1.51 | 6.31 ± 1.57 | 1.80 (0.402 to 3.212) | 0.014 |
| MSM (mg/kg/min) | 3.51 ± 0.65 | 2.44 ± 0.90 | 1.07 (0.352 to 1.775) | 0.006 |
| Steady-state insulin (mU/l) | 31.98 ± 7.86 | 34.45 ± 8.46 | − 2.47 ( − 9.922 to 4.986) | 0.497 |

Abbreviations: CI, confidence interval; MSM, *M*-value adjusted to total skeletal muscle mass calculated using arm, thigh and calf muscle circumferences; *M*-value$_{corrected}$, *M*-value after space correction.

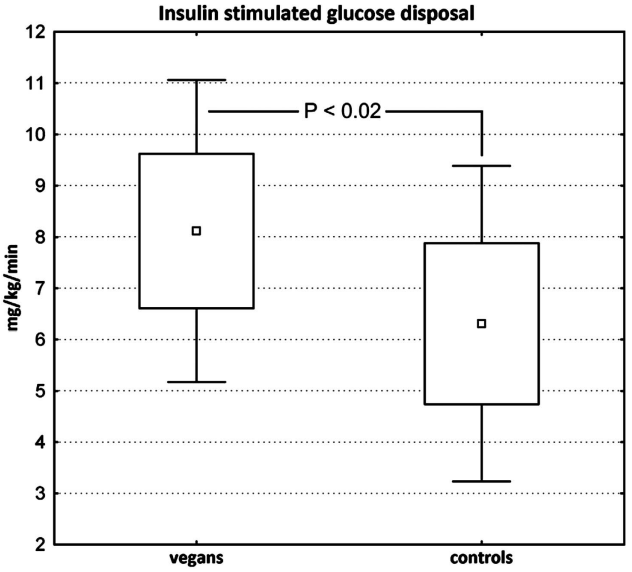


**Figure 1.** Insulin-stimulated glucose uptake. Comparison between vegans and controls. Results are expressed in mg/kg/min, 95% confidence interval for difference: 0.402–3.212.

steady-state insulinemia, which was sufficient to suppress endogenous insulin production (data not shown).

### Mitochondrial density and IMCL content

Results are summarized in Table 4. Markers of mitochondrial density (CS activity and relative amount of mtDNA to nuclear DNA) did not show significant differences, although there was a trend toward a higher relative mtDNA content in vegans (0.23, $P = 0.135$). There was also an apparent trend toward a lower IMCL content in vegans ( − 3.45 mg/g, $P = 0.193$). Vegans had lower amounts of pentadecanoic ( − 0.04 mg/g, $P = 0.004$) and docosahexaenoic (DHA; − 0.08 mg/g, $P = 0.003$) in muscle.

### Correlations

We analyzed correlations of data pooled from all subjects and found that plasma insulin levels were negatively correlated with mtDNA amount ($R = 0.55$, $P = 0.01$) and there were trends toward a negative correlation between glycemia and mtDNA ($R = 0.30$, $P = 0.180$), a positive correlation between *M*-values and mtDNA ($R = 0.30$, $P = 0.191$), a positive correlation between the Baecke score and mtDNA ($R = 0.34$, $P = 0.07$) and a positive correlation between CS and mtDNA ($R = 0.33$, $P = 0.15$). Surprisingly, in vegan sample the *M*-value was highly positively correlated with IMCL content ($R = 0.85$, $P < 0.001$).

## DISCUSSION

In this study, we investigated the influence of a vegan diet on IS and phenomena associated with IR in skeletal muscle, particularly mitochondrial density. It has been previously established that vegans are more insulin sensitive than omnivores and have a lower IMCL content.[1–3] We hypothesized that the reported higher IS and lower IMCL content in vegans are associated with a higher mitochondrial density. A major finding of the current study is that vegans are more insulin sensitive, but we found only statistically insignificant differences in IMCL content or mitochondrial density compared with omnivores.

### Glucose homeostasis

We found that vegans have lower fasting plasma glucose and insulin levels, and higher insulin-stimulated glucose disposal. This is a plausible explanation for the lower incidence of IR-related diseases in vegans and similar data have been previously published.[1,2] However, to our knowledge the current study was the first to use hyperinsulinemic euglycemic clamp for the assessment of IS.

### Plasma lipid and FFA profile

A survey of the literature shows that vegans have more favorable lipid profiles, have lower levels of total and LDL plasma cholesterol and lower triglycerides.[3,25] Our results showed significantly lower total cholesterol levels in vegans and we found that vegans have higher plasma levels of total polyunsaturated fatty acids and higher amounts of omega-6 fatty acids linoleic acid, eicosadienoic acid and dihomo-γ-linolenic in their FFA profiles. We also found higher plasma levels of omega-3 ALA in vegans, which has not been previously reported. Published studies suggest that vegans have lower circulating EPA and DHA levels.[26] Indeed, vegan's intake of EPA and DHA is very low and, therefore, these FA are mainly synthesized endogenously from ALA. Unfortunately, we cannot comment on EPA and DHA levels in circulation because the detection limit of the method used in this study for EPA and DHA was 1.0 and 2.2 μg/ml, respectively, and none of the subjects in our study exceeded these limits.

### IMCL content

Previous studies reported that IMCL content correlates with IR in healthy non-diabetic subjects[9] and that vegans have lower IMCL values compared with omnivores.[2] However, a lower IMCL content in vegans was found only in the (oxidative) soleus muscle and not in the (glycolytic) tibialis anterior muscle. We obtained samples from the vastus lateralis, which is glycolytic, and we did not find any statistically significant differences among the groups even though there was a trend toward a lower IMCL content and a positive correlation between IMCL content and glucose disposal (*M*-value) in vegans. Biochemical methods of IMCL detection are burdened by potential contamination with subcutaneous and

**Table 4.** Mitochondrial density and IMCLs

| | *Vegans* (n = *11*) | *Controls* (n = *10*) | *95% CI for difference* | P-*value* |
|---|---|---|---|---|
| CS (μmol/g/min) | 18.43 ± 5.05 | 18.16 ± 5.41 | 0.27 (−4.503 to 5.050) | 0.906 |
| mtDNA | 1.36 ± 0.31 | 1.13 ± 0.36 | 0.23 (−0.078 to 0.537) | 0.135 |
| *Muscle lipids (mg/g of muscle)* | | | | |
| Total IMCL | 13.91 (7.8–44.0) | 17.36 (12.4–78.5) | −3.45 (−7.594 to 24.550) | 0.193 |
| SFA | 4.34 (2.9–9.8) | 5.58 (3.8–21.8) | −1.24 (−1.299 to 6.895) | 0.149 |
| MUFA | 4.60 (1.2–21.1) | 6.59 (2.2–43.7) | −1.99 (−3.969 to 15.484) | 0.342 |
| PUFA | 7.02 ± 3.22 | 6.94 ± 2.60 | 0.08 (−2.616 to 2.772) | 0.952 |
| PDA (C15:0) | 0.01 (0.01–0.04) | 0.05 (0.01–0.19) | −0.04 (−0.085 to −0.014) | 0.004 |
| DHA (C22:6n3) | 0.09 ± 0.05 | 0.17 ± 0.05 | −0.08 (−0.126 to −0.031) | 0.003 |

Abbreviations: CI, confidence interval; CS, activity of citrate synthase; mtDNA, relative copy number of mtDNA counted as fractional ratio mtDNA/ncDNA; total IMCL, total intramyocellular lipids; PDA, pentadecanoic; DHA, docosahexaenoic; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

extramyocellular fat tissue, and therefore we performed a microdissection of the muscle samples immediately after biopsy, using a binocular microscope to minimize this potential confounder. The advantage of a biochemical assessment of IMCL is the possibility to measure individual FFA content in the lipid extract. We found that vegans had lower DHA in their muscles compared with omnivores, although the ALA and EPA content was similar (data not shown). As already mentioned, circulating EPA and DHA levels are lower in vegans due to their lower intake.[26] To our knowledge, this study is the first to show a decreased DHA content in muscle tissue samples. The clinical impact of the previously reported lower circulating EPA and DHA levels and lower muscle DHA content in vegans deserves further investigation.

### Mitochondrial density

Mitochondrial aerobic capacity in skeletal muscle is potentially a major contributor to whole-body IS with skeletal muscles responsible for almost 85% of insulin-stimulated glucose uptake.[8] It was previously reported that insulin-resistant subjects have a lower mitochondrial oxidative capacity[13,27] and lower mitochondrial content.[12,13] There are currently no published data addressing the effect of a vegan diet on mitochondrial density in skeletal muscle. We assessed the mitochondrial density by measuring CS activity (nuclear-encoded mitochondrial enzyme) and relative amounts of mtDNA in skeletal muscle. There was a trend toward a higher relative amount of mtDNA in vegans and a correlation between mtDNA and CS, which would be in line with literature,[28] but these differences did not reach statistical significance. The relative amount of mtDNA was negatively correlated with plasma insulin levels and there was a trend toward a positive correlation between mtDNA and glucose disposal (*M*-value) across the whole sample. This suggests that a reduction in IS and the related elevation of plasma insulin levels may be linked with decreased amounts of mtDNA and a possible loss of mitochondrial oxidative capacity.

### Limitations of this study

The main limitation of this study was its relatively small sample size. Because of strict inclusion criteria, we were not able to enroll more eligible subjects into the study. Groups were intentionally matched so that the maximum number of variables associated with IR could be controlled. In spite of the intention to match groups in baseline characteristics, there were small differences between groups, namely in age, visceral and subcutaneous fat and physical activity. We cannot exclude that these differences could have had some influence on results. Vegans were on average 2.51 years older than omnivores in our sample. However, we do not believe this age difference had a significant effect on the tested variables, as both groups were relatively young.

Physical activity and $VO_2$max has an important role in the development of IR, as well as in muscle lipid accumulation and mitochondrial biogenesis. Physical activity represents a major potential confounder when it comes to the development of IR. Therefore, the aim of the initial matching of groups was to use the same level of physical activity between groups. In this study, $VO_2$max was not assessed and the Baecke questionnaire of habitual physical activity was used instead. Although $VO_2$max is a more accurate parameter for describing physiological changes induced by physical activity, it has been shown that Baecke questionnaire scores correlate well with $VO_2$max.[19]

### Conclusion

We found that vegans have more favorable glucose homeostasis and plasma lipid profile, which is in line with previous studies. We also found that vegans have higher plasma levels of polyunsaturated fatty acid, more precisely linoleic acid, ALA, eicosadienoic acid and dihomo-γ-linolenic, and lower skeletal muscle DHA content. We also demonstrated that vegans had a significantly higher insulin-stimulated glucose uptake; however, we found only small or no differences in IMCL, mtDNA and CS activity compared with their matched omnivorous counterparts. These findings suggest that a decrease in whole-body glucose disposal precedes muscle lipid accumulation and mitochondrial bioenergetic failure in the development of IR. Therefore, IMCL accumulation and mitochondrial dysfunction may be consequences or epiphenomena associated with IR, and may contribute to its progression rather than the initial trigger.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

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**Article 4**

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

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

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# Normalizing Glutamine Concentration Causes Mitochondrial Uncoupling in an In Vitro Model of Human Skeletal Muscle

**Adela Krajcova, MD[1]; Jakub Ziak[1]; Katerina Jiroutkova, MD[1]; Jana Patkova[1]; Moustafa Elkalaf, MD[1]; Valer Dzupa, MD, PhD[2]; Jan Trnka, MD, PhD[1]; and Frantisek Duska, MD, PhD[1]**

## Abstract

*Background*: Glutamine has been considered essential for rapidly dividing cells, but its effect on mitochondrial function is unknown. *Materials and Methods*: Human myoblasts were isolated from skeletal muscle biopsy samples (n = 9) and exposed for 20 days to 6 different glutamine concentrations (0, 100, 200, 300, 500, and 5000 μM). Cells were trypsinized and manually counted every 5 days. Seven days before the end of exposure, half of these cells were allowed to differentiate to myotubes. Afterward, energy metabolism in both myotubes and myoblasts was assessed by extracellular flux analysis (Seahorse Biosciences, Billerica, MA). The protocol for myoblasts was optimized in preliminary experiments. To account for different mitochondrial density or cell count, data were normalized to citrate synthase activity. *Results*: Fastest myoblast proliferation was observed at 300 μM glutamine, with a significant reduction at 0 and 100 μM. Glutamine did not influence basal oxygen consumption, anaerobic glycolysis or respiratory chain capacity. Glutamine significantly ($P = .015$) influenced the leak through the inner mitochondrial membrane. Efficiency of respiratory chain was highest at 200–300 μM glutamine (~90% of oxygen used for adenosine triphosphate synthesis). Increased glutamine concentration to 500 or 5000 μM caused mitochondrial uncoupling in myoblasts and myotubes, decreasing the efficiency of the respiratory chain to ~70%. *Conclusion*: Glutamine concentrations, consistent with moderate clinical hypoglutaminemia (300 μM), bring about an optimal condition of myoblast proliferation and for efficiency of aerobic phosphorylation in an in vitro model of human skeletal muscle. These data support the hypothesis of hypoglutaminemia as an adaptive phenomenon in conditions leading to bioenergetic failure (eg, critical illness). (*JPEN J Parenter Enteral Nutr*. 2015;39:180-189)

## Keywords

extracellular flux analysis; human myoblasts; bioenergetic failure; aerobic phosphorylation; glutamine

## Clinical Relevancy Statement

Hypoglutaminemia is a feature of a wide range of wasting diseases, and glutamine supplementation as a part of nutrition support seemed to be beneficial in most.[1] However, recent work suggests that critically ill patients with multiorgan failure do not benefit[2] or are harmed[3] by aggressive glutamine supplementation. Mitochondrial dysfunction present in critical illness (and absent in most other diseases) may be the key factor interfering with glutamine effects and explaining the differences in clinical outcome. In this study, we look, for the first time, at the interaction between extracellular glutamine concentration and mitochondrial function in human skeletal muscle cells. We demonstrate that at moderate hypoglutaminemia (300 μM), myoblast proliferation is fastest and adenosine triphosphate synthesis is most efficient, while severe hypoglutaminemia (<100 μM), normal (500 μM) or supranormal (5000 μM) glutamine levels lead to respiratory uncoupling and energy wasting in an in vitro model of human skeletal muscle.

## Introduction

Muscle mass regeneration, during recovery from wasting illnesses, is vitally dependent on the function of myoblasts.[4] These cells are generated by proliferation of normally quiescent satellite cells, which are attached to the muscle basement membrane. After stimulation by anabolic signals, satellite cells start to proliferate and differentiate to myoblasts. Myoblasts

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synthesize contractile proteins and mitochondria and, finally, fuse with muscle fibers. Differentiated cells ready for fusion with muscle fibers are called myotubes. Myotubes have been used as an in vitro model of human skeletal muscle, since their metabolic features are similar[5] to in vivo muscle fibers. As most rapidly dividing cell lines use glutamine as both nitrogen donor and energy substrate,[6] it could be hypothesized that hypoglutaminemia impairs the proliferation and energy metabolism of myoblasts. This could provide a possible mechanism of enhanced lean body mass recovery with glutamine supplementation seen in some trials,[7] since glutamine does not influence protein synthesis directly.[8] In this study, we ask how hypoglutaminemia, at the degree seen in catabolic illnesses, influences the rate of proliferation and energy metabolism in human myoblasts and myotubes. Cells were isolated from muscle biopsy samples and their energy metabolism was assessed after 20 days of exposure 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[9]), and a concentration commonly used in cell cultures (5000 μM[6,10-15]).

## Methods

### *Overview of Study Design*

See Figure 1. For each of 9 participants, we first isolated satellite cells from a vastus lateralis biopsy sample and created a primary culture of myoblasts. At the beginning of experiment (day 0), we divided cells into 6 groups and exposed them in duplicates to 6 different glutamine concentrations. Cells were trypsinized, manually counted, and reseeded at days 5, 10, 15, and 20. This allowed us to create proliferation curves. A subset of cells was reseeded at day 15 into a 24-well Seahorse plate (Seahorse Biosciences, Billerica, MA) and allowed to differentiate into myotubes during next 7 days. At day 20, myoblasts were seeded into another Seahorse plate. In both Seahorse plates, cells were still exposed, in tri- or tetraplicates, to 6 different glutamine concentrations before extracellular flux analyses were performed on both myoblasts (n = 8) and myotubes (n = 7). Seahorse plates were kept frozen for later analysis of protein content and citrate synthase activity in a subset of patients (n = 5 and 4 for myoblasts and myotubes, respectively).

### *Study Participants and Muscle Biopsy*

Note: Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (St Louis, MO).

For all studies, we used muscle biopsy samples obtained from patients (n = 9) undergoing elective hip replacement surgery at the Department of Orthopaedic Surgery of Kralovske Vinohrady University Hospital in Prague. The University Hospital Ethical Committee reviewed both the protocol and consent form and approved the study. All patients provided written prospective informed consent. We excluded the patient with diabetes mellitus. Muscle biopsies were taken from the vastus lateralis muscle by an open surgical technique (~300 mg) as soon as the muscle was exposed during surgery. Biopsy samples were collected into 5 mL of cold Dulbecco's modified Eagle's medium (DMEM) with glucose (5.56 mM), pyruvate (1 mM), and phenol red (GIBCO, Carlsbad, CA) supplemented with glutamine (5000 μM), penicillin-streptomycin solution (100 IU/mL–100 μg/mL), and fungizone (0.5 μg/mL) and immediately put on ice.[16]

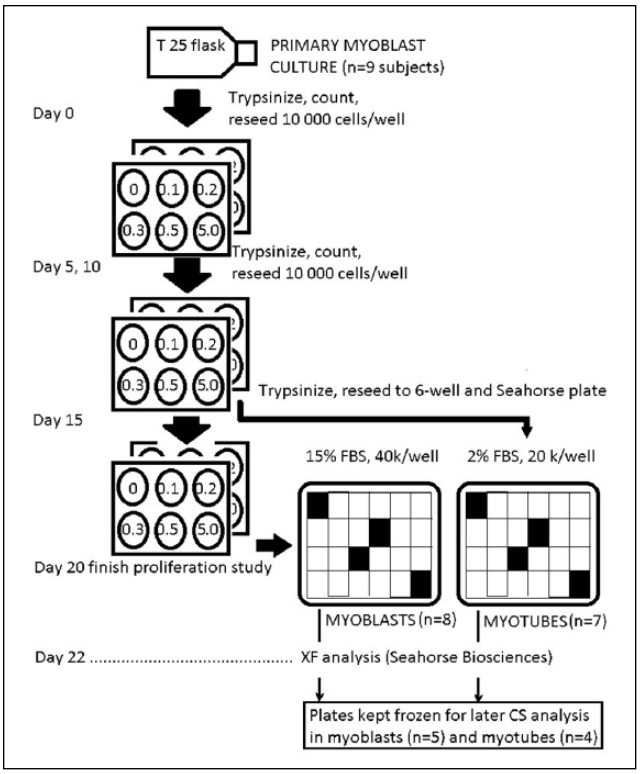


**Figure 1.** Schematic representation of the study design. CS, citrate synthase; FBS, fetal bovine serum.

### *Isolating and Culturing Human Myoblasts*

Satellite cells were isolated from muscle tissue as previously described,[17] with a few modifications. At least 150 mg of skeletal muscle tissue was minced and washed by Hank's balanced salt solution 2–3 times and cells were subsequently dissociated by incubation in 0.25% Trypsin/0.68% collagenase solution in a shaking water bath at 37°C for 30 minutes. Fetal bovine serum (FBS) was added to the suspension, and cells were collected by centrifugation at 350 *g*. Cells were subsequently preplated in an uncoated Petri dish for 60 minutes at 37°C with 5% $CO_2$ to remove fibroblasts and then transferred to gelatin-coated flasks (T-25) in DMEM with glucose (5.56 mM), pyruvate (1 mM), and phenol red (GIBCO). The medium was

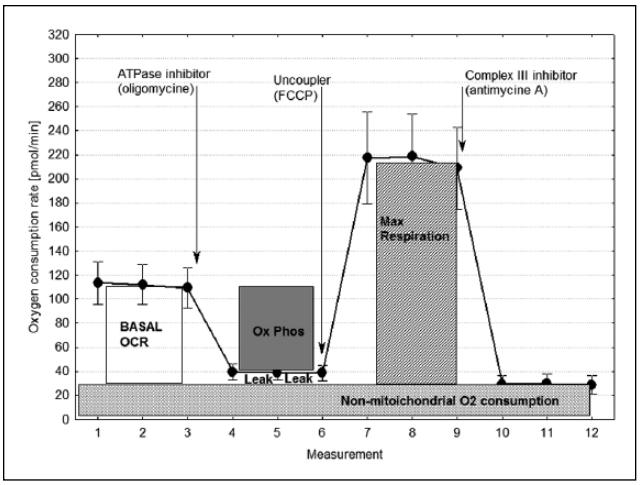


**Figure 2.** Principle of extracellular flux analysis of mitochondrial metabolism. Pooled data from myoblasts of all participants were used to construct this graph. Means, vertical bars represent 95% confidence intervals. FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; OCR, oxygen consumption rate; Ox Phos, oxidative phosphorylation. See text for details.

supplemented with 15% FBS, penicillin-streptomycin solution (100 IU/mL–100 µg/mL), fungizone (0.05 µg/mL), dexamethasone (0.4 µg/mL), fetuin (50 ng/mL), insulin (10 µg/mL), human growth factors (epidermal growth factor [EGF], 10 ng/mL; fibroblast growth factor [FGF], 1 ng/mL), and 5 mM glutamine. The plates were cultured at 37°C in 5 $CO_2$. The media were changed every 2 or 3 days until cells reached 80%–90% confluence.

## *Proliferation Rate Measurement*

Myoblasts from the primary culture were trypsinized and cells were seeded into a 6-well plate in basal growth medium–DMEM with glucose (5.56 mM) and pyruvate (1 mM) (GIBCO) supplemented with 15% FBS, penicillin-streptomycin solution (100 IU/mL–100 µg/mL), fungizone (0.05 µg/mL), dexamethasone (0.4 µg/mL), fetuin (50 ng/mL), insulin (10 µg/mL) and human growth factors (EGF, 10 ng/mL; FGF, 1 ng/mL) with 0, 110, 240, 370, 640, or 5000 µM glutamine, respectively. When preparing fresh media for all but the 5000-µM glutamine groups, we considered glutamine content in FBS and spontaneous glutamine breakdown with a half-time of 3 days at 37°C.[18] After solving the first-order kinetic equation ($C = C_0 \cdot e^{-kt}$, where C is the concentration at time t = 18 hours [half of the media-change interval], $C_0$ is the concentration at the beginning, and k is the first-order rate constant—in our case, 0.0104 $h^{-1}$) and subtracting the amount of glutamine in 15% FBS (17 µM[19]), the concentrations of glutamine in the middle of the media exchange interval should be 0, 100, 200, 300, and 500 µM. The 5000-µM group had 5000 µM in fresh media, since this is routine laboratory practice, and even after partial degradation, glutamine still remains highly redundant. The medium was changed every 2 or 3 days. At days 5, 10, and 15, cells were trypsinized, counted and reseeded. At day 20, cells were trypsinized, counted and seeded in a 24-well gelatin-coated Seahorse plate for flux analysis. As a measure of proliferation rate, we calculated the number of cells produced from a single cell during 5 days of culture. This number was calculated by dividing the number of cells at the end of the 5-day interval by the number of cells seeded into the well (ie, by 10,000).

*Cell viability* was measured using the CellTiter96 MTS assay (Promega, Madison, WI) as previously described.[20] Viable cells reduce the tetrazolinum dye, MTS, to purple formazan. The absorbance at 490 nm is then used as a measure of cell viability. We performed MTS on myoblasts from primary culture at baseline and after 20 days of exposure to different glutamine levels.

## *Extracellular Flux Analysis*

We used a Seahorse XF24 Analyzer (Seahorse Biosciences) for all experiments. The principle of the method is described elsewhere.[21] In brief: oxygen consumption rate (OCR) is measured in a microlayer, which sits above cells seeded in a 24-well microplate. Four wells were used as background control, with the remaining 20 wells for testing cells at 6 glutamine concentrations in tri- to tetraplicates. The measurement is performed at baseline (phase 1) and after the addition of up to 4 test agents. The dynamics of OCR allows the assessment of mitochondrial function. In our experiments, we used the sequence of an ATPase inhibitor oligomycine (phase 2), followed by an inner membrane uncoupler, FCCP (carbonyl cyanide-4-[trifluoromethoxy]phenylhydrazone, phase 3). Finally, we blocked the respiratory chain complex III with Antimycin A (phase 4). Oxygen consumption after exposure to Antimycin A is considered nonmitochondrial (see Figure 2). Basal OCR was calculated as $OCR_{Phase1} - OCR_{Phase\ 4}$. When comparing OCR before-and-after exposure with inhibition, the absolute cell number is irrelevant since the same population of cells is compared. Therefore, most parameters of energy metabolism are expressed as a percentage of baseline value. *ATP turnover* was calculated as $100*(OCR_{Phase1} - OCR_{Phase2})$/Basal OCR [%]. *Leak* through the inner mitochondrial membrane was calculated as $100*(OCR_{Phase2} - OCR_{Phase4})$/Basal OCR [%]. By analogy, *respiratory chain capacity* (or maximum respiration) was calculated as $100*(OCR_{Phase3} - OCR_{Phase4})$/Basal OCR. Extracellular acidification rate (ECAR) relates to lactate production[21] and is used as a measure of the rate of anaerobic glycolysis. Seahorse measurements were performed for myoblasts from 8 participants and myotubes from 7 participants.

To reflect possible differences of cell number or mitochondrial content, we normalized basal OCR to the activity of citrate synthase (CS) in a subset of participants (n = 5 for myoblasts and n = 4 for myotubes).

### Protein Content and CS Activity

Immediately after XF flux measurement, each well in a plate was washed with cold phosphate-buffered saline (PBS) and filled with 150 µL M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA) with 2 µL Protease inhibitor cocktail. Then the plate was stored at –80°C. On the day of the assay, plates were thawed and the cellular lysate was transferred into Eppendorf tubes and centrifuged at 15,000 *g* for 15 minutes at 4°C. The supernatant was collected for protein determination and CS activity assay. For protein determination, we used the Bradford reagent according to the manufacturer's instructions. CS activity was assayed in myoblasts from participants 2, 3, 4, 5, and 6 and myotubes from participants 1, 3, 5, and 6 using a CS assay kit (Sigma-Aldrich) with at least 8 µg of whole-cell extract protein, 0.3 mM acetyl-CoA, and 0.1 mM 5,5′-dithiobis-(2-nitro-benzoic acid). The reaction was started with adding 5 mM oxaloacetate to the mixture. CS activity of cells is stable 2–3 hours during storage on ice.[22] Enzyme activity was monitored by recording the changes in absorbance at 412 nm over 1.5 minutes at 30°C.

### Optimizing the XF Flux Analysis Protocol for Human Myoblasts

In a series of preliminary experiments, we first optimized the conditions of the measurement for human myoblasts by modifying existing protocols for C2C12 myoblasts[23] and human myotubes.[14] We started with a protocol for C2C12 myoblasts seeded at 20,000 cells/well, realizing that maximum OCR reached only the median of 76 or 65 pM/min after 1 or 2 µM FCCP, respectively, while OCR for the same number of C2C12 cells was approximately 10 times higher. In subsequent experiments, we increased the number of cells seeded per well and compared 20,000, 40,000, and 75,000 cells/well. With 40,000 cells, we achieved maximum OCR well above 100 pmol/min, while $pO_2$ remained above 5 kPa throughout the experiment. In control wells (without addition of inhibitors), OCR remained stable during 240 minutes of the experiment. We next compared the effects of 0.76, 1.0, and 1.5 µM oligomycine, achieving maximum inhibition of ATPase with 1.0 µM. This concentration was therefore used for the rest of the experiments. Similarly, we compared uncoupling effects of 0.5, 1, and 2 µM FCCP and achieved maximum OCR with 1 µM. Last, to verify the ability of 4 µm Antimycin A to inhibit the respiratory chain, we performed an experiment in which we added 0.6 M KCN (complex IV inhibitor) after Antimycin A. No additional decrease in OCR was seen with the addition of cyanide. In light of this, the final protocol we used for testing the influence of glutamine on mitochondrial function uses 40,000 cells/well in basal growth medium with 0, 100, 200, 300, 500, or 5000 µM glutamine. After 24 hours, we exchanged the medium for XF Assay Medium (Seahorse Biosciences) with 4 mM glucose and 1 mM pyruvate (pH 7.4) at 37°C. XF flux analysis was performed after a 60-minute incubation in a $CO_2$-free atmosphere. Baseline OCR was measured in 3 cycles: 3 minutes mixing, 2 minutes waiting and 3 minutes measuring. Then these 3 cycles were repeated after adding oligomycin (1 µM), FCCP (1 µM), and Antimycin A (4 µM).

### XF Flux Analysis of Human Myotubes

After 15 days of exposure to various glutamine concentrations, cells were trypsinized and seeded on a 24-well gelatin-coated Seahorse plate at 20,000 cells/well in a basal growth medium with 6 different concentrations of glutamine. After 24 hours, we exchanged the medium for a differentiation medium (DMEM) with glucose (25 mM), pyruvate (1 mM), 2% horse serum, penicillin-streptomycin solution (100 IU/mL–100 µg/mL), insulin (10 µg/mL), and the respective glutamine concentrations. Medium was exchanged every 2 days for the next 6 days, and on day 7, an XF assay was performed as described[14]; assay medium and concentrations of uncoupler and inhibitors were identical to those used for myoblasts.

### Statistics

All data were tested for normality of distribution. We used 1-way or factorial analysis of variance (ANOVA, with Tukey post hoc test where appropriate) for most comparisons. Proliferation data (number of daughter cells, N) were found to be log-normally distributed, and after logarithmic transformation, they were fitted with the normal distribution ($P > .2$ in the Kolmogorov-Smirnov test) and processed by factorial ANOVA. All calculations were performed in Statistica version 8 (StatSoft, Inc, Tulsa, OK). Differences at $P < .05$ were considered significant.

## Results

### Fastest Cell Proliferation Was Observed in 300 µM Glutamine

Cells across a range of glutamine concentrations showed exponential proliferation (see Figure 3), which was fastest in 300 µM, as demonstrated by mean numbers of cells formed from 1 ancestor cell during a 5-day cycle of culture (geometric means of all 4 culture intervals): 10 (0 µM glutamine), 15 (100 µM), 18 (200 µM), 25 (300 µM), 24 (500 µM) and 23 (5000 µM). Across all glutamine concentrations, we observed the proliferation rate to slow down after 10 days. However, cell viability as per the MTS test was not compromised ($P = .86$, data not shown). Influences of time and glutamine concentration were both significant at $P < .001$ in factorial ANOVA, but there was no interaction between them ($P = .83$).

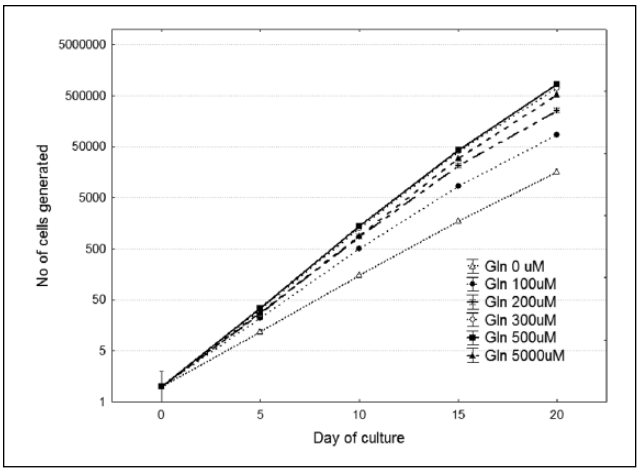


**Figure 3.** Proliferation rate of human myoblasts in relation to glutamine (Gln) concentration in the media. Mean number of cells formed from a single cell at day 0. Note log scale; confidence interval bars are not displayed for clarity.

## *Glutamine Deprivation Does Not Influence Mitochondrial Density but Tends to Decrease Protein Content*

Protein content was higher in wells containing myotubes, despite the fact that half the number of cells were seeded (20,000 myotubes and 40,000 myoblasts per well). In both cell lines, total glutamine deprivation tended to decrease total protein content per well by up to ~50% (Figure 4A) compared with cells cultured in 5000 μM glutamine. There was also a significant correlation between protein content in a well and glutamine in the media (Spearman $R = 0.46$ for myotubes and $R = 0.38$ for myoblasts; $P < .05$ for both). No such trend was seen for CS activity, which remained unchanged across glutamine concentrations (Figure 4B), and the differences in CS activity between cell lines roughly correspond with the fact that twice as many myoblasts were seeded per well compared with myotubes.

In turn, CS per protein ratio (a measure of relative mitochondrial density) tended to increase with severe glutamine deprivation (Figure 4C) as well as OCR/ECAR ratio (Figure 4D), reflecting the relative contributions of aerobic and anaerobic metabolism on ATP generation. Cells cultured in a standard "laboratory" concentration of glutamine (5000 μM) were highly oxidative, having an OCR/ECAR ratio of 11 ± 4 (myoblasts) or 9 ± 2 (myotubes). Severe glutamine deprivation tended to increase the OCR/ECAR ratio even further, reaching 28 ± 10 or 19 ± 9, respectively, in cells cultured in glutamine-free media.

## *Glutamine Does Not Influence Basal OCR*

After normalization to mitochondrial content (expressed as CS activity), basal respiration does not differ either between myoblasts and myotubes ($P = .53$) or across glutamine concentrations ($P = .87$) (see Figure 5A).

## *Influence of Glutamine on the Efficiency and Capacity of the Respiratory Chain*

After subtracting nonmitochondrial oxygen consumption, OCR at baseline reflects a sum of oxygen consumed for ATP synthesis and for energy dissipation due to leak of protons through the inner mitochondrial membrane. These 2 components are distinguished by measuring OCR after the addition of the F1F0 ATPase inhibitor oligomycine (Figure 2). The ATP synthesis rate normalized to mitochondrial content (CS activity) was not different either between cell lines ($P = .16$) or across glutamine concentration ($P = .63$; see Figure 5C). Leak of protons through the inner mitochondrial membrane normalized to CS activity (Figure 5D) was significantly higher in myotubes compared with myoblasts ($P < .001$) and was influenced by glutamine concentrations ($P = .041$). The relation of glutamine concentration to mitochondrial leak was further explored by looking at that percentage of basal OCR, which is not inhibited by oligomycine (Figure 6).

Myotubes cultured at 200–300 μM glutamine used 80%–90% of basal OCR for ATP synthesis and only 10%–20% of energy was dissipated as heat. At both extremes of glutamine concentration, we observed increased mitochondrial uncoupling to ~30%–40%, which decreased efficiency of the respiratory chain below 70% (see Figure 6). Myoblasts had significantly lower leak compared with myotubes ($P = .019$), which only increased with high glutamine concentrations (from ~10% to ~30%). Even though the effect of glutamine was significant in the overall model ($P = .015$), none of the differences were significant in the Tukey post hoc test (eg, $P = .053$ for the difference between 200 and 5000 μM in myotubes).

Spare respiratory chain capacity (maximum respiration) is measured as oxygen consumption after artificial uncoupling of the inner mitochondrial membrane (refer to Figure 1). If expressed as OCR normalized to mitochondrial content (Figure 5B), maximum respiration tends to be nonsignificantly higher in myoblasts compared with myotubes ($P = .13$), and it is not influenced by glutamine ($P = .94$). Similarly, when related to basal respiration, maximum respiration is 200%–300% and not different between cell lines or among glutamine concentration.

## *No Influence of Glutamine on Anaerobic Glycolysis, Glycolytic Capacity, or Nonmitochondrial Oxygen Consumption*

ECAR is used as a measure of anaerobic glycolysis as it mainly reflects the production of lactate.[21] There was no difference in anaerobic glycolysis in a range of glutamine concentrations in

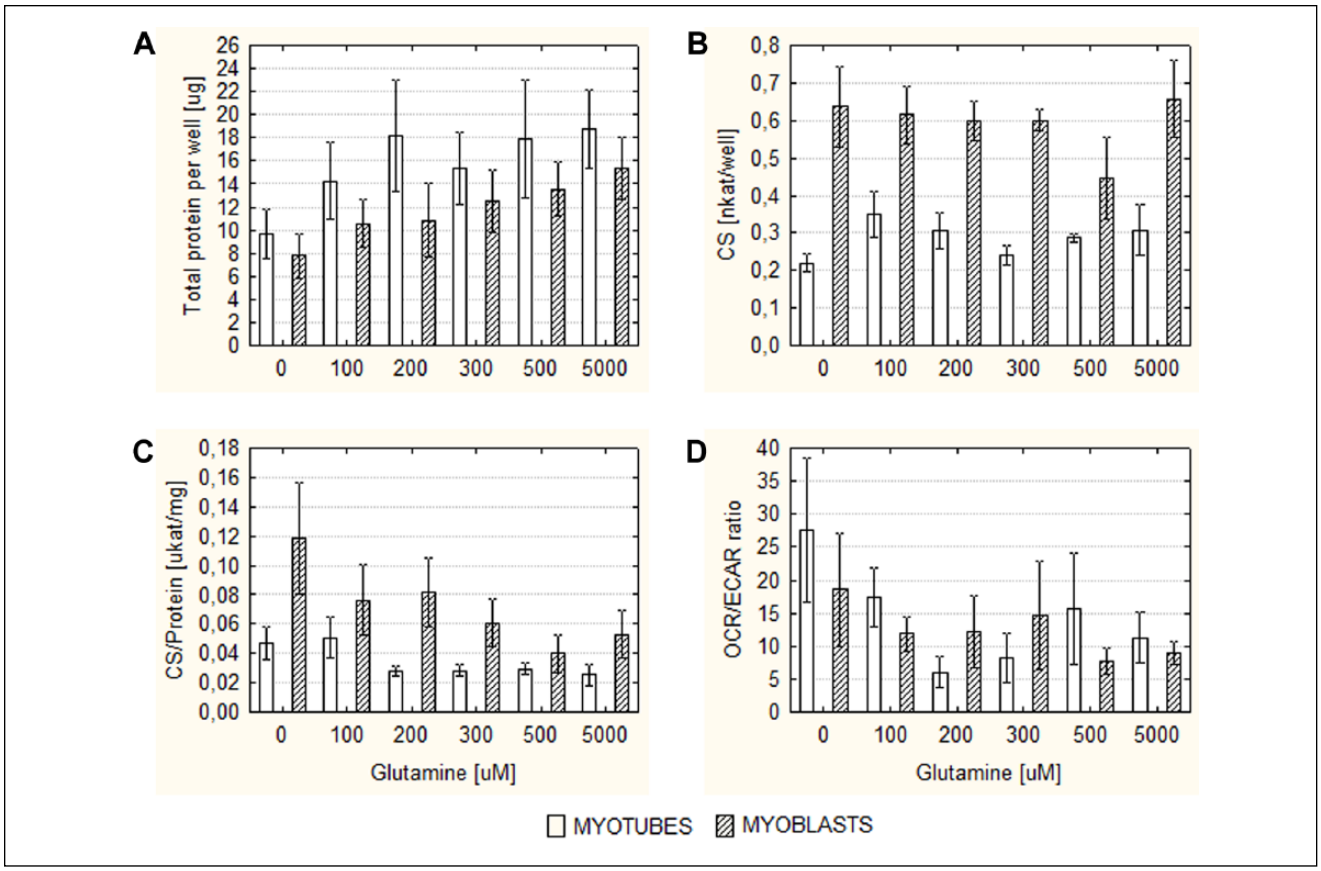


**Figure 4.** (A) Total protein content per well. (B) Citrate synthase (CS) activity per well. (C) CS per protein ratio. (D) Oxygen consumption rate (OCR) per extracellular acidification rate (ECAR) ratio. Means, vertical bars represent 95% confidence intervals.

either cell lines. After blocking ATP synthase with oligomycine, anaerobic glycolysis becomes the only source of ATP for the cell, and hence it increased to ~110%–170% above baseline. This response was unaffected by glutamine concentration, and neither was extra-mitochondrial oxygen consumption (data not shown).

### *Overall Metabolic Profile of Myoblasts and Myotubes in Relation to Glutamine in Culture Media*

Last, to get further insight into energy metabolism, we expressed oxidative phosphorylation, anaerobic glycolysis, and energy dissipation via proton leak in equivalents of ATP and compared those values (Figure 7). We assumed that 1 mol of $O_2$ of oligomycine-inhibitable OCR gives rise to 5 mol of ATP and that 1 mol of $H^+$ (or 1 mol of lactate) produced by cells equates to the synthesis of 1 mol of ATP.

Figure 7 again demonstrates the highly oxidative nature of both myotubes and myoblasts and the tendency of increased leak with higher glutamine concentrations, particularly in myotubes. ATP wasted by inner mitochondrial leak is not compensated by increased anaerobic glycolysis. Of note, these data were not normalized to CS activity (as doing so would make no sense for anaerobic metabolism), and therefore it is not possible to compare myotubes with myoblasts or perform any valid statistical analysis.

## Discussion

Rapidly dividing cells have been shown to be dependent on glutamine as a substrate for de novo nucleotide synthesis[24] and energy metabolism.[10,24,25] This has been demonstrated for lymphocytes,[26] enterocytes[27] and type II pneumocytes.[28] This study, for the first time, shows a similar dependency for human myoblasts. Surprisingly, supra-physiological concentrations of glutamine (5000 μM) as recommended by many protocols for in vitro cell cultures,[6,10-13] including human myoblasts,[14] do not bring any additional benefit in terms of myoblast proliferation. Furthermore, it may even be the case that concentrations as low as 300 μM glutamine, which is close to the concentration in patients with protracted critical illness in the absence of glutamine supplementation,[29] are optimal. Significant limitations of myoblast proliferation were observed at concentrations

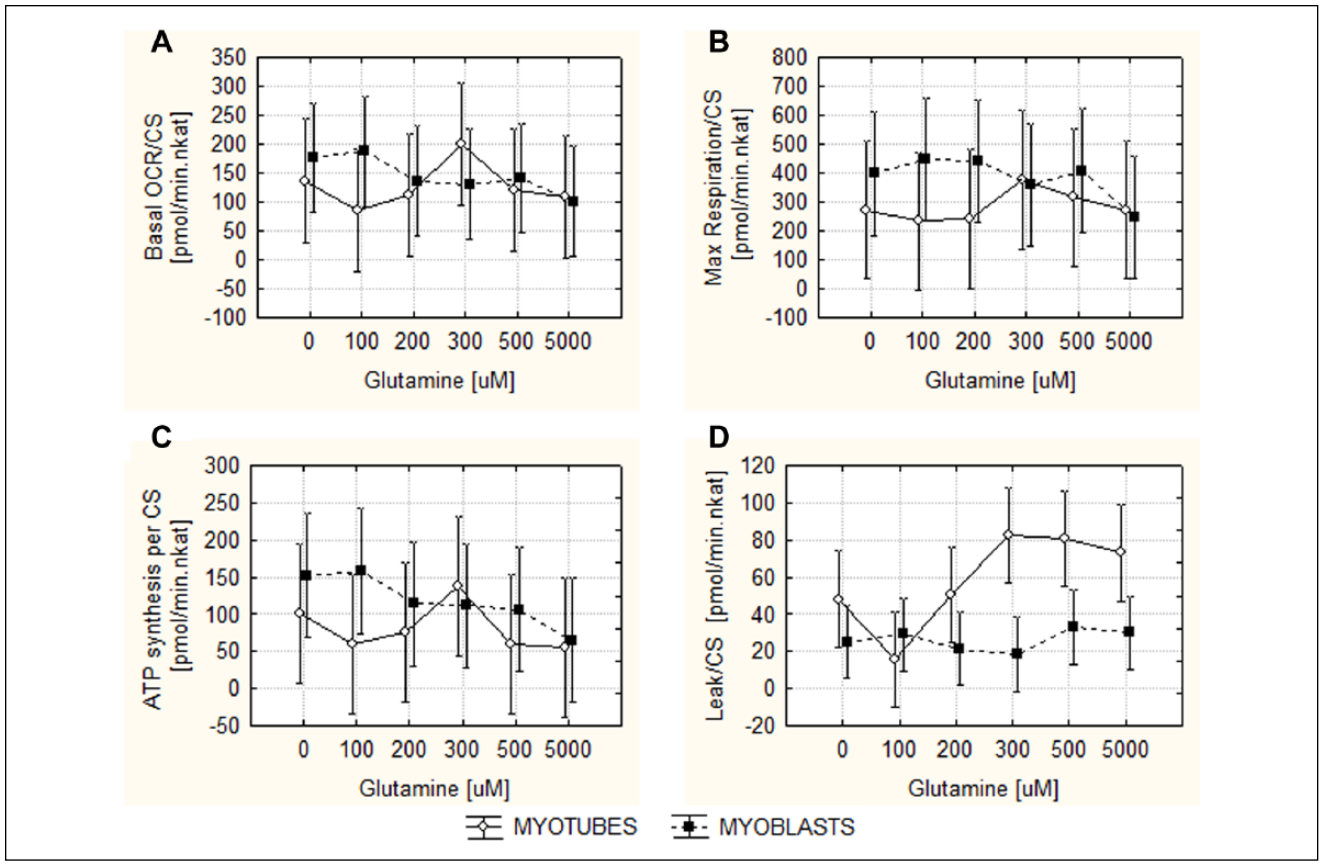


**Figure 5.** (A) Basal oxygen consumption rate. (B) Maximal respiration. (C) Adenosine triphosphate (ATP) synthesis rate. (D) Proton leak through the inner mitochondrial membrane. All values are expressed as the amount of oxygen used after adjustment to mitochondrial content in a well expressed as citrate synthase (CS) activity (pmol/min$^{-1}$/nkat$^{-1}$). Means, vertical bars represent 95% confidence intervals. OCR, oxygen consumption rate.

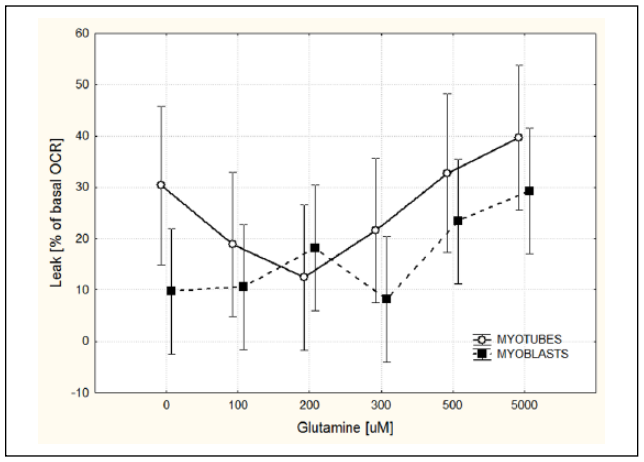


**Figure 6.** Proton leak through the inner mitochondrial membrane expressed as a percentage of the basal oxygen consumption rate. Means, vertical bars represent 95% confidence intervals. OCR, oxygen consumption rate.

≤200 μM glutamine, which is well below the range seen in wasting diseases[30] or in acute critical illness.[29,31,32] Although it is difficult to extrapolate in vitro data to whole-body physiology, our results suggest that restoring extracellular glutamine concentration to normal levels may not improve conditions for muscle regeneration. Even cells grown in glutamine-free media remained viable and did proliferate (Figure 3). A trend to a decrease in protein content per well (Figure 4A) without a change in CS activity (Figure 4B) in wells seeded with glutamine-deprived cells may reflect a lower number of cells (with more mitochondria), diminished total protein content in the same number of cells, or a combination of both. Nicklin et al[33] demonstrated, in various mammalian cell lines, that glutamine is an upstream activator of mTOR, a key regulator of protein synthesis.[34] This could represent a molecular mechanism as to how glutamine deprivation can alter intracellular protein content. Because the differentiation stage may vary among different glutamine concentrations, total protein content may not represent cell number, and we did not use it for normalization of OCR in metabolic studies. Instead, we adjusted OCR to CS activity—a marker of mitochondrial density.[22]

Myoblasts and myotubes cultured in a standard "laboratory" concentration of glutamine (5000 μM) were largely reliant on aerobic phosphorylation, having an OCR/ECAR ratio

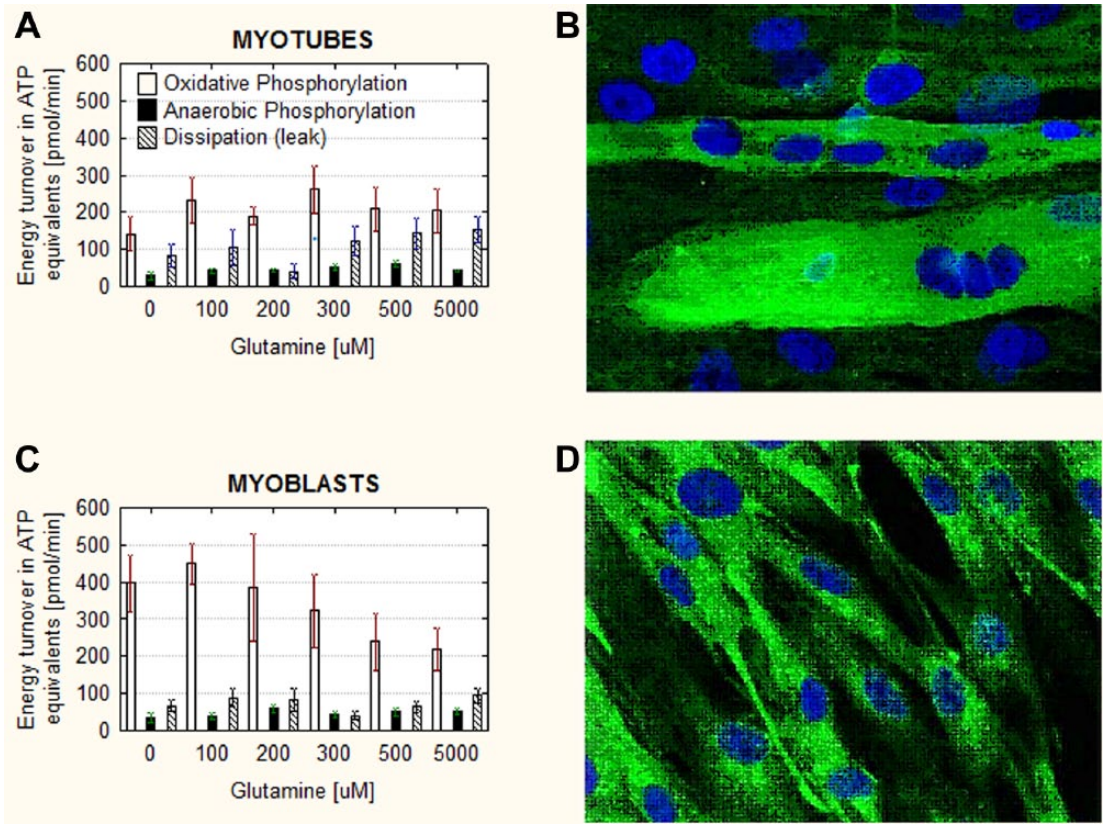


**Figure 7.** Energy profile (A, C) and the appearance of myotubes (B) and myoblasts (D) stained for desmin (AB 907 rabbit anti–desmin polyclonal antibody [Millipore, Billerica, MA]). See text for explanation. ATP, adenosine triphosphate.

~10, which is comparable to feline cardiomyocytes[35] or C2C12 myoblasts.[36] Much lower OCR/ECAR (typically between 1 and 5) ratios are reported for most cancer cell lines.[37] Figure 7 demonstrates a very small contribution of anaerobic phosphorylation to overall ATP turnover. A trend to a further increase of the OCR/ECAR ratio in glutamine-deprived cells may reflect the fact that pyruvate—instead of being converted to lactate—is turned into oxaloacetate. This anaplerotic reaction can replenish intermediates of the citric acid cycle when there is not enough glutamine to form 2-oxoglutarate via glutamate. However, we failed to demonstrate the inhibitory effect of glutamine deprivation on anaerobic glycolysis either at baseline (Figure 7) or after inhibition of aerobic ATP synthesis.

When looking at the effect of glutamine on energy metabolism of myotubes and myoblasts, the most interesting finding is the uncoupling effect of glutamine. Oxygen used in respiratory chain energizes both ATP synthesis and proton leak through the inner mitochondrial membrane. In both myoblasts and myotubes cultured at 200–300 μM glutamine, we observed highly coupled respiration with ~90% of basal OCR being used to drive ATP synthesis (Figures 5D and 6). Supra-physiological concentrations of glutamine (5000 μM) decreased the efficiency of the respiratory chain to ~60%–75% in both cell lines. In myotubes but not myoblasts, we saw a similar degree of uncoupling with extremely low glutamine (0 μM). When looking at absolute numbers, the uncoupling did not compromise aerobic ATP generation (Figure 5C) or alter basal OCR (Figure 5A), even though some trends to decrease aerobic phosphorylation are seen in myoblasts (Figures 5A and 7). Mitochondrial uncoupling can result either from inner mitochondrial membrane damage or controlled leak of protons through the family of uncoupling proteins (UCPs). Of these, UCP-2 and UCP-3 are found in skeletal muscle.[38] In cultured macrophages, colonocytes, and pancreatic β-cells, glutamine has been shown to increase translation of UCP-2 with the maximum induction seen at 0.4–1.0 mM.[39] It has been proposed[40] that UCP-3 and UCP-2 may also serve as uniporters of pyruvate from—and glutamine into—mitochondria, respectively. In this context, the need to transport abundant glutamine into mitochondria to join the citric acid cycle (via glutamate and 2-oxoglutarate) may have induced UCPs and caused the uncoupling seen with higher glutamine concentrations. Skeletal muscle is the main organ for glucose disposal in vivo, and glutamine-induced uncoupling should then increase substrate metabolism and energy expenditure. In line with this, glutamine supplementation in critically ill patients has been repeatedly shown to

increase insulin-mediated glucose disposal[41-43] and fat oxidation.[44] Iwashita et al[45] observed increased energy expenditure with glutamine in healthy volunteers. In this context, with glutamine supplementation and uncoupling, glucose and fat can be increasingly oxidized in skeletal muscle to energize futile cycles and heat generation.

However, in our study, we have not demonstrated any significant increases in basal OCR with glutamine-induced uncoupling, and it seems that increased leak was rather at the expense of aerobic phosphorylation (albeit its changes were not significant). This would suggest a rather uncontrolled leak resulting from glutamine-induced mitochondrial damage. Possible mitochondrial toxicity of glutamine was first noted by Groening et al.[46] When investigating the protective effect of glutamine on the activity of cytochrome c oxidase in septic rat myocardium, they found a trend to decreased oxygen extraction and diminished cyclooxygenase (COX) activity in sham-operated (nonseptic) hearts, despite higher COX levels with 0.75 g/kg glutamine compared with placebo. The authors mention the possibility of glutamine intracellular breakdown to glutamate and ammonia, which resulted in oxidative damage of mitochondria.[46]

It should be noted that muscle biopsies were taken from participants with hip arthropathy, which may have influenced muscle physiology. The culture media with different glutamine concentrations were not isonitrogenous, and we cannot rule out the possibility that the observed effects are due to different nitrogen content rather than glutamine itself. Moreover, the methods used do not allow deeper insight into observed changes of energy metabolism. Further studies should explore the effects of glutamine on intracellular ATP concentration, inner mitochondrial membrane potential, and expression of UCPs, as well as measure mitochondrial DNA oxidative damage in human nontumor cell lines. If "mitochondrial toxicity" of glutamine is confirmed, this would shed new light onto recent data from large randomized controlled trials on glutamine supplementation. Glutamine seems to be beneficial in chronic wasting diseases but harmful in acute critical illness with multiorgan failure.[3] Our data generate the hypothesis that the reason for the discrepancy of clinical outcomes with glutamine may lie in mitochondria: bioenergetic failure is a feature of critical illness, where altered and swollen mitochondria are not able to meet extreme ATP demands of tissues, leading to multiorgan failure and death. The hypoglutaminemia of critical illness may represent an adaptive phenomenon[47] by decreasing circulating mitochondrial toxin below what we consider safe levels. This may be protective of mitochondria at the expense of other glutamine-dependent processes. After the initial insult and following mitochondrial damage and repair, the beneficial effects of higher levels of glutamine may prevail, since these other glutamine-dependent processes may become more important in the subsequent rehabilitation phase. During the recovery phase after mitochondria are repaired and replenished in survivors[48] and also in chronic illnesses, the beneficial effect of glutamine may prevail.

In conclusion, in this study, we have developed a protocol for the use of extracellular flux analysis in cultured human myoblasts. We have shown that human myoblast proliferation is optimal at 300 μM glutamine, which is a level consistent with moderate clinical hypoglutaminemia. Increasing glutamine concentration above this level does not improve the proliferation rate and leads to significant uncoupling of the respiratory chain and a trend to impaired ATP synthesis in both myoblasts and myotubes. Glutamine does not influence respiratory chain capacity, the rate of anaerobic glycolysis, or non-mitochondrial oxygen consumption in human skeletal muscle cells.

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# 4 CONCLUSIONS

The results presented in this thesis contribute to the understanding of the effects of selected nutrients (i.e. different fatty acids and glutamine) and mechanisms of their action on a cellular level. 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 function, 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 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. Exposure to unsaturated FFA, but not saturated PA, caused mild uncoupling of mitochondrial respiration. In studies concerning the effects of glutamine we found that levels of glutamine consistent with moderate clinical hypoglutaminemia (300 μM) 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 and decreased efficiency of oxidative phosphorylation.

We also showed that metabolic benefits of 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 [153,156]. We observed increased levels of plasma PUFA and lower DHA content in skeletal muscles of vegans compared with omnivore controls.

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 is affected also by glutamine. Several new questions have arisen from our results, which may hopefully serve as the basis for future studies.

# 5 ANNEXES

List of articles not included in the thesis:

**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. 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.

**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

**4. 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. Feb 21. [Epub ahead of print]. IF 2.72

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