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List of Abbreviations

$\Delta\psi_m$	electrical membrane potential difference between mitochondrial matrix and cytoplasm
ECAR	extracellular acidification rate
OCR	oxygen consumption rate
TMRM	tetramethylrhodamine methyl ester
TPMP	methyltriphenylphosphonium
TPP ⁺	triphenylphosphonium moiety

1 Summary

Skeletal muscle is the largest tissue in the body and plays a marked role in the homeostasis of the body metabolic state. Mitochondria have been proven to contribute to the pathophysiology of various metabolic diseases, either due to defects in the bioenergetic properties or production of the reactive oxygen species. In this work murine myoblasts C2C12 were used as a model of skeletal muscle *in vitro*, and rat muscle was used to prepare homogenate enriched in the mitochondrial fraction.

This work investigates the changes in respiratory parameters in models where mitochondrial oxidative phosphorylation is induced by changing the available consumable substrates in the culture media, such as replacing glucose by galactose, and the effect of treating the cells with high glucose concentration during the process of differentiation on mitochondrial performance. It also investigates the changes in bioenergetic profiles in samples treated with inactive derivatives of the widely used triphenylphosphonium (TPP⁺) salts to target mitochondria by various probes and antioxidants.

The methods used in this study included evaluating mitochondrial parameters in intact and permeabilized cells by real time measurement of the oxygen consumption rate using the extracellular flux analyzer, spectrophotometric measurement of the enzymatic activity of Krebs cycle and the electron transport chain, and fluorometric measurement of changes in mitochondrial membrane potential ($\Delta\psi_m$).

The results confirmed that low glucose concentration is the main inducer of mitochondrial respiration and changes observed with galactose-treated models are due to glucose deprivation. The presence of glucose in the culture media is essential to induce differentiation and increasing the glucose level during the myogenic process decreases in the respiratory capacity due to the decrease in the enzymatic activity of complex I and III. More hydrophobic long alkyl side chain of the TPP⁺ derivatives induces mitochondrial uncoupling and proton leak respiration, while the least hydrophobic methytriphenylphosphonium (TPMP⁺) causes gradual decrease of mitochondrial respiration by interruption of Krebs cycle and inhibition of oxoglutarate dehydrogenase complex.

2 Souhrn (Kindly translated by Veronika Šrámková)

Kosterní svalovina, jakožto nejobjemnější tkáň v těle, má nezastupitelnou úlohu při udržování metabolické homeostázy. Bylo dokázáno, že mitochondrie přispívají k patofyziologii nejruznějších metabolických onemocnění, ať již kvůli poškození bioenergetických vlastností nebo produkcí reaktivních forem kyslíku. V této práci byly jako *in vitro* model kosterního svalu použity myší myoblasty C2C12 a sval křesy, ze kterého byl připraven homogenát obohacený o mitochondriální frakci.

Cílem práce bylo stanovit změny v mitochondriálních respiračních parametrech daných dostupností využitelných substrátů v kultivačním mediu, např. nahrazením glukózy galaktózou, a také zjistit vliv vysokých koncentrací glukózy na mitochondriální aktivitu během diferenciací. Dalším cílem bylo objasnit efekt inaktivních derivátů trifenyl fosfoniových (TPP^+) solí, hojně využívaných pro doručení různých průběhů a antioxidantů do mitochondrií, na bioenergetický profil buněk.

Data byla získána pomocí metod umožňující měření spotřeby kyslíku v reálném čase na extracelulárním flux analyzátoru a to jak v buňkách s neporušenou membránou, tak v permeabilizovaných buňkách, dále pomocí spektrofotometrického měření enzymatické aktivity Krebsova cyklu a elektronového transportního řetězce, a nakonec pomocí fluorometrické detekce změn membránového potenciálu mitochondrií ($\Delta\psi_m$).

Výsledky potvrzují, že nízká koncentrace glukózy je hlavním spouštěčem mitochondriálního dýchání a že změny pozorované u modelů kultivovaných s galaktózou jsou způsobeny nedostatkem glukózy. Přítomnost glukózy v kultivačním mediu je tedy nezbytná pro indukci diferenciací a zvyšování hladiny glukózy během myogenního procesu vede k poklesu respirační kapacity následkem snížení enzymatické aktivity komplexu I a III. Dlouhý alkylový postranní řetězec TPP^+ derivátů, který se vyznačuje silnějšími hydrofobními vlastnostmi, indukuje mitochondriální odpřažení a únik protonů, zatímco nejméně hydrofobní metyl(trifenyl)fosfoniová sůl (TPMP^+) způsobuje postupný pokles aktivity mitochondriální respirace přerušením Krebsova cyklu a inhibicí komplexu oxoglutarát dehydrogenázy.

3 Introduction

The present work is concerned with the study of energy metabolism in skeletal muscle, particularly the mitochondrial respiratory function and the changes in respiratory parameters corresponding to changes in the available consumable substrates. In studies examining *in vitro* models, the composition of growth media is usually considered as a background condition, which has no significant effect on observed phenomena. Proper controls are usually considered to elucidate the response to novel treatments or concentrations, disregarding the contribution of media in reforming the bioenergetic characterization of the tested model, which complicates viewing of final conclusions with objective detachment.

One of the major complications facing *in vitro* studies is the decrease in the reliance on mitochondria oxidative phosphorylation to provide energy. Cells cultured in media with standard concentrations of glucose tend to acquire highly glycolytic phenotypes (“Crabtree effect” [1]), despite the presence of satisfactory levels of oxygen, which makes them less suitable as models for metabolic studies aiming to test mitochondrial respiration. Attempts have been made to overcome this phenomenon, by substituting glucose for galactose, which does not support anaerobic glycolysis. This is usually explained by the fact that galactose cannot be oxidized to pyruvate without prior conversion to glucose, which consumes two molecules of ATP, thus making anaerobic glycolysis insufficient to produce energy. Galactose-fed cells then should rely on mitochondrial oxidative phosphorylation to produce ATP, hence providing researchers with a metabolically improved model for studying mitochondrial respiratory function. Several studies performed on various tissues and cell lines have shown substantial changes in energy metabolism under such conditions where galactose-based media are often recommended to circumvent the Crabtree effect [2-5].

The use of galactose supplemented cell culture media instead of glucose, although being simple, is exposing the cultured cells to glucose deprivation, which is another metabolic factor that participates in the observed results of the previous studies. Hence it was a necessity to differentiate the changes caused by using galactose from

that resulted from glucose fasting. Moreover, the use of galactose with tissues such as skeletal muscle is questionable, due to the lack of decisive amount of information about galactose metabolism in skeletal muscle cells. On the other hand, it is necessary to provide a suitable model of laboratory cultured skeletal muscle where the cultured cells possess more tendency to produce energy by mitochondrial oxidative phosphorylation pathway for a relevant investigation of mitochondrial performance, and describe an optimal substrate concentration to support cellular growth and differentiation, inclusive of testing the response of cultured muscle cells to different concentrations of the available substrates.

In this work I tried to perform a detailed study on using different substrates that can drive the cells towards a less glycolytic behavior, and analyze the mitochondrial membrane properties in response to changes of media substrate concentration, and I provide evidence arguing against the use of galactose instead of glucose to enhance mitochondrial respiration and increase the reliance on oxidative ATP production. I also compare the bioenergetic profiles of different metabolic phenotypes as well as the cellular behavior and ability to differentiate in the presence or absence of glucose, to demonstrate the cause of previous studies published observations that used galactose and documented alterations in mitochondrial respiration in skeletal muscle were substantially due to glucose deprivation but not because of galactose metabolism.

I devoted a part of the study to present a comparison between hyperglycemic and normoglycemic environmental changes in mitochondrial respiratory parameters of *in vitro* muscle cells grown and differentiated in either case, and I will provide data supporting that high glucose levels may eventually result in a mitochondrial respiratory dysfunction and the possible characterization of the lower respiratory capacity associated with the constant presence of high glucose level.

The final part of this work includes a detailed study about the adverse effects of the lipophilic triphenylphosphonium (TPP⁺) compounds [6–8], the most used mitochondrial targeting moieties. I used moieties conjugated to inactive alkyl side chains to focus the comparison on the degree of the lipophilic character, mediated by the length

of the alkyl side chain. I will provide data confirming interaction of the highly lipophilic alkylTPP⁺ compounds with mitochondrial inner membrane enzymatic function, and draw the attention to the adverse inhibition of Krebs cycle by the least hydrophobic moiety methyltriphenylphosphonium (TPMP⁺).

4 Aim of this work

The project was designed to investigate the energy metabolism of skeletal muscle in various metabolic conditions, which provided freedom in selecting research subjects. Mitochondria, as the main energy producing organelles, and changes in mitochondrial activity due to tissue culture conditions provided an opportunity to study skeletal muscle metabolism *in vitro*.

As our understanding of the effects of low glucose and galactose on cultured skeletal muscle cells is still far from satisfactory, the first intention was to investigate the changes of growth patterns and several parameters of mitochondrial metabolism in C2C12 myoblasts and myotubes in response to differing availability of glucose or galactose while considering some of the shortcomings of previously published studies in an attempt to avoid them. The goal was to provide experimental data demonstrating all aspects of the relationship between cell culture conditions and the metabolic activity of the cultured cells.

Another aim was to analyze the changes and if possible the deterioration in mitochondrial respiratory parameters of cultured cells in a high glucose concentration imitating uncontrolled hyperglycemia, and to compare the mitochondrial performance with cells cultured in moderate glucose concentration.

While the main target was to study metabolism, using different probes to study mitochondrial function opened an additional area of mitochondrial research, and the subject of adverse reactions of mitochondrial targeting molecules attracted increasing attention. This allowed to investigate the effect of the length of the alkyl side chain of the hydrophobic TPP⁺ moieties on mitochondrial respiration, and try to answer the questionable TPMP⁺ inhibitory effect.

5 Experimental procedures

C2C12 murine cells were used in this work as an *in vitro* model of skeletal muscle. Both undifferentiated and differentiated forms were used according to the experimental settings. The enzymatic activity of the respiratory enzymes was assayed using samples that included isolated mitochondria from C2C12 cells and rat skeletal muscle homogenate enriched in the mitochondrial fraction.

Analysis of metabolism of intact and permeabilized cells was performed using the extracellular flux analyzer XF-24, which allowed a simultaneous measurement of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in real-time [9,10]. Metabolic differences were demonstrated by using a sequence of respiratory inhibitors and detecting the changes in OCR and ECAR values. The results obtained from these measurements were used to conclude the bioenergetic profile of the tested cells, in the form of several respiratory parameters such as ATP-driven respiration and respiratory capacity.

This technology was also employed to estimate the free palmitate oxidation in intact C2C12 myotubes. By selectively permeabilizing the plasma membrane of intact cells using the XF plasma membrane permeabilizer (Seahorsebio), different mitochondrial substrate were used to investigate different respiratory enzymes.

Differences in respiratory parameters were further investigated by measuring the activity of different respiratory enzymes spectrophotometrically, such as the pyruvate dehydrogenase complex, krebs cycle enzymes and enzymes of the electron transport chain.

The detection of the changes in mitochondrial membrane potential ($\Delta\psi_m$) was measured fluorometrically, using the negatively charged dye tetra methylrhodamine methyl ester (TMRM), and semi-qualitative changes in $\Delta\psi_m$ were concluded as changes in the fluorescent intensity. Measurements took place using Leica TCS SP II confocal microscope and FACSCalibur flow cytometer.

Other used methods included the measurement galactose level in the culture media and also the detection of myogenic differentiation markers such as MyoD and myosin heavy chain by immunofluorescent staining.

6 Results and Discussion

6.1 Low glucose but not galactose enhances oxidative respiration

Previous studies on murine myoblasts showed that despite the availability of oxidizable fuels in the incubation medium, myoblasts in the early stage of differentiation derive approximately 60% of their energy demands by lactate production from glucose [11]. For that reason attempts to develop a more oxidative model were made either by lowering the glucose level [12] or by substituting galactose for glucose [5] where Aguer et al. presented data showing that the differentiation of human primary myoblasts in a galactose-containing medium reveals mitochondrial dysfunction in samples derived from formerly diabetic patients, who lost weight and became normoglycemic. However, the authors compared cellular respiration in media with different compositions thus making it difficult to distinguish acute effects of substrate availability from longer-term phenotypic changes in cells grown in galactose-containing medium.

Previous publications demonstrated the inability of skeletal muscle to utilize galactose as a fuel [13, 14], and the results of chapter 3 confirm these reports. The first finding was that growing myoblasts do not utilize available galactose in the medium and had a growth rate indistinguishable from cells grown in a carbohydrate-free environment. More evidence was obtained by testing the ability to differentiate depending on galactose as a source of energy. A significant limitation of the galactose culture medium for C2C12 myoblasts is presented by the observation that they fail to differentiate in the absence of glucose, which is in good agreement with previous studies on glucose deprivation [15, 16].

The bioenergetic profile of the cells grown on galactose and that deprived of glucose showed a great similarity. Glucose deprivation caused a significant decrease in the respiratory capacity. In addition, glucose level was found to change the mitochondrial activity in cells grown on high concentration of glucose (5 g/l). The cells grown on moderate glucose concentration (1 g/l) were found to be an optimal respiratory phenotype of C2C12.

6.2 High glucose induces mitochondrial dysfunction in differentiated muscle cells

The detrimental effects of the persistent exposure to high glucose level in the extracellular environment can be found clinically in uncontrolled diabetes. The relation between mitochondrial dysfunction and the development of diabetes has been a subject of extensive research. It has been suggested that insulin-resistant develops due to defects in mitochondria function, that leads to insufficient ATP production for the hexokinase as well as other reactions requiring phosphorylation [17]. Mitochondrial dysfunction in diabetic cases was manifested as impaired mitochondrial capacity for fat oxidation during fasting conditions [18].

Consequences of sustained high glucose level *in vivo* is highly complicated due to the interaction with manifestations and/or causes of diabetes and insulin resistance. Therefore, testing simply the effect of high glucose environment on mitochondrial performance *in vitro* may contribute partially in interpreting an aspect of the intricate figure found in diabetes.

The results discussed in chapter 4 show that incubating and allowing the C2C12 cells to differentiate in a high level of glucose (25 mM) led to the development of mitochondrial dysfunction, manifested by a lower respiratory capacity of the high glucose treated phenotype when compared to the cells differentiated in a normoglycemic environment.

Although the mitochondrial yield was significantly decreased in the high glucose treated cells, the citrate synthase activity was not different. This finding directed the study to explore the mitochondrial respiratory chain activity, which revealed a significant decrease in the activity of complex I and complex III, while other complexes were not affected.

The trial to investigate the ability to oxidize free fatty acids, such as palmitate, encountered a difficulty due to the uncoupling effect of the free palmitic acid. The difference in utilizing exogenous palmitate between the two phenotypes was not distinguishable. Both phenotypes responded equally to palmitate addition by increasing their respiration, however this increase was caused by an increase in the leak respiration.

6.3 Adverse effects of the highly lipophilic triphenylphosphonium cations

The accumulation of the lipophilic TPP⁺ derivatives in mitochondria was first described in 1970 [19, 20] where it was suggested to be used to measure mitochondrial membrane potential using mainly methyltriphenylphosphonium salts. Since their discovery and the lipophilic cations based on the triphenylphosphonium moiety have been widely used to target various biologically active substances such as antioxidants [21, 22], spin traps [23, 24] or various other chemical probes into mitochondria [25, 26].

Assuming a perfectly Nernstian behavior, a membrane-permeable cation will accumulate in a negatively charged compartment approximately ten-fold for each 60 mV of potential difference. In the case of TPP⁺ derivatives, this ideal behavior is complicated by the fact that the hydrophobicity of the derivative affects both the extent and the rate of accumulation [27, 28].

The results of chapter 5 explains the previously published reports using different cell lines [29]. The nearly similar response of intact cells to alkylTPP⁺ is useful to identify broad effects on mitochondrial bioenergetics, however, additional investigations were performed to pinpoint more precise mechanisms of action of the TPP⁺ derivatives.

The assumed biologically ‘inactive’ hydrophobic alkylTPP⁺ derivatives caused a remarkable increase in proton leak respiration and a significant decrease in the respiratory capacity in intact C2C12 cells. The spectrophotometric assays of the enzymatic activity of the respiratory chain complexes in tissue homogenate enriched in mitochondrial fraction confirmed direct non-specific inhibition of all complexes by the longer-chain derivatives. In either case, the response to hydrophobic alkylTPP⁺ derivatives was dose-dependent.

Independent measurement of mitochondrial membrane potential ($\Delta\psi_m$) in intact C2C12 showed that there is a clear trend towards lower fluorescence intensities as the alkyl chain length increases. This data matched perfectly with the results obtained from the metabolic analysis where the lipophilic TPP⁺ salts enhanced proton leak respiration. In addition, the inhibitory effect on respiratory chain activity was demonstrated.

6.4 Methyltriphenylphosphonium targeting of 2-oxoglutarate dehydrogenase complex

Methyltriphenylphosphonium (TPMP⁺) is the most used alkylTPP⁺, and its main application is the direct measurement of mitochondrial membrane potential, using the TPMP⁺-sensitive electrodes [30, 31]. Its inhibitory effect when used with high extra-mitochondrial concentrations was reported in studies with isolated mitochondria [32, 33]. Unlike the highly lipophilic alkylTPP⁺ derivatives, A direct interference with the mitochondrial membrane potential is most unlikely to be the reason of TPMP⁺ inhibitory effect. A possible explanation of this effect can be demonstrated by recognizing the available binding sites where TPMP⁺ molecules may exert an adverse reaction.

The data of chapter 6 confirms the inhibitory effect of TPMP⁺ on cellular respiration. Intact C2C12 cells decreased gradually the reliance on oxidative respiration and shifted immediately towards glycolytic respiration. In permeabilized cells, the inhibition was restricted to NADH-linked substrates, while the respiration on succinate was mildly affected .

Spectrophotometric measurements of the enzymatic activity of mitochondrial respiratory enzymes in the presence of 1 mM TPMP⁺ (approximate equivalent of 1 μ M extracellular concentration) showed that the activity of 2-oxoglutarate dehydrogenase complex (OGDH) complex was effectively reduced. The activity of other enzymes including the structurally similar pyruvate dehydrogenase complex was not affected or non significantly reduced. The IC₅₀ of TPMP⁺ was estimated to be 3.93 [3.70-4.17] mM and other enzymes were resistant to TPMP⁺ with a minor non significant reduction in the activity of complex I.

The OGDH activity was increasingly affected by the length of the alkyl side chain of the alkylTPP⁺. This raises a question about the structure of the enzyme and the possible attraction force to more harmful hydrophobic probes. This inhibition can lead to accumulation of some of the citric acid cycle intermediates, which as a consequence may influence intracellular signaling and gene expression via the role of 2-oxoglutarate in prolyl hydroxylation of HIF-1 alpha and other signaling processes [34].

7 Conclusions

Galactose is not a suitable fuel for skeletal muscle The use of galactose with skeletal muscles was proved in chapter 3 to be inappropriate. C2C12 cells do not utilize galactose even when cultured in glucose free media. The growth rate of cells treated with galactose was similar to those deprived of glucose, and both phenotypes possessed a significant lower growth rate than glucose fed cells. The level of media galactose was not changed when incubated with the cultured cells. The undifferentiated myoblasts failed to differentiate when supplemented with galactose and the presence of glucose is essential for C2C12 to differentiate. Cells lack of glucose failed to express the myosin heavy chain differentiation marker, however, evidence of activation of the differentiation pathway was observed by expressing the MyoD transcriptional factor.

Oxidative respiration is enhanced by lowering glucose The results of chapter 3 also show that using galactose or no glucose in the cell growth medium fails as a simple method to enhance the oxidative metabolism of C2C12 cells. Observable changes in mitochondrial respiratory parameters associated with the use of galactose were basically due to glucose deprivation. The effects of glucose deprivation are complex and depend, among other things, on the cell type used. For C2C12 cells, these results support a recommendation to use a moderate glucose concentration for cultivation and avoid high-glucose growth media. Different culture media may have a significant effect on the capacity of the mitochondrial respiratory chain, but the link of such a change to the extent of oxidative metabolism of the cultured cells remains unclear. In addition mitochondrial mass markers were not different among all groups of cells, and so were the glycolytic capabilities of the cells treated with normal, high or complete absence of glucose.

Glucose level optimization is essential to reveal the variation between different bioenergetic profiles Assessment of mitochondrial respiratory parameters showed a marked variation when the glucose level was changed. Differences in respiratory ca-

capacity were best observed when the assay condition included 1 g/l glucose. When assessed in a glucose free medium supplemented with the mitochondrial substrates pyruvate and glutamine, the previously observed differences were masked. These observations recommend the use of a unified medium with identical composition and supplements for a proper comparison of mitochondrial parameters among different phenotypes.

High glucose decreased mitochondrial respiratory capacities The data obtained from chapter 4 confirms the direct relation between high glucose level in the culture media and the development of mitochondrial dysfunction. Allowing the C2C12 cells to grow and differentiate in a high glucose environment caused a lower respiratory capacity when compared to myotubes treated with normal glucose concentration. The differences includes also a lower mitochondrial mass yield, and lower activity of complex I and complex III. The differences did not include any change in glycolytic profiles. A major limitation to test the ability to oxidize free palmitic acid is due to the uncoupling effect of palmitate, which resulted in a large increase in leak respiration.

The hydrophobicity of mitochondrial targeting molecules negatively affect respiratory efficiency The widely used mitochondriotropic triphenylphosphonium (TPP⁺) derivatives were shown to interfere with mitochondrial bioenergetics in chapter 5. Although facilitating mitochondrial targeting, high hydrophobicity of the TPP⁺ molecule alters the bioenergetic performance by markedly increasing the proton leak respiration and significantly decrease the coupling efficiency in intact cells. In isolated mitochondrial preparations, the more hydrophobic TPP⁺ moieties inhibited the enzymatic activity of the mitochondrial respiratory chain. The mechanism of inhibition is not completely revealed, and disruption of the mitochondrial phospholipid membrane remains the most plausible explanation, due to the non-specific inhibition on all respiratory complexes. The hydrophobic mitochondrial targeting molecules are recommended to be tested for the adverse respiratory inhibition when designing new ones for use as diagnostic probes or therapeutic agents.

Methyltriphenylphosphonium inhibits Krebs cycle Another adverse response of TPP⁺ moieties is discussed in chapter 6. The least hydrophobic alkylTPP⁺, methyltriphenylphosphonium (TPMP⁺), inhibits mitochondrial respiration by directly inhibiting Krebs cycle. It selectively targets the 2-oxoglutarate dehydrogenase complex and interferes with the complex function, which declined markedly when treated with more hydrophobic alkyl TPP⁺ compounds. The IC₅₀ of TPMP⁺ is 3.93 mM in isolated mitochondrial fractions (3.93 μM extra-cellular). The enzyme 2-oxoglutarate dehydrogenase complex showed less activity when treated with more hydrophobic alkylTPP⁺ derivatives, which draws the attention towards the importance of this enzyme, and also towards the distribution and binding of mitochondrial targeting molecules to mitochondrial matrix and membrane structures.

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List of publications

7.1 In relation to the thesis

Elkalaf M¹, Andel M, Trnka J (2013) Low Glucose but Not Galactose Enhances Oxidative Mitochondrial Metabolism in C2C12 Myoblasts and Myotubes. PLoS ONE 8(8): e70772. *IF 3.534*

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