

Aspalathin Alleviates Skeletal Muscle Insulin Resistance and Mitochondrial Dysfunction

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Summary

Natural compounds may bear promising therapeutic benefits against metabolic diseases such as type 2 diabetes mellitus (T2DM), which are characterized by a state of insulin resistance and mitochondrial dysfunction. Here, we examined the cellular mechanisms by which aspalathin, a dihydrochalcone C-glucoside unique to rooibos, may ameliorate palmitate-induced insulin resistance and mitochondrial dysfunction in cultured C2C12 myotubes. This current study demonstrated that aspalathin remains effective in improving glucose uptake in insulin-resistant skeletal muscle cells, supported by the upregulation of insulin-dependent signaling that involves the activation of insulin receptor (IR) and direct phosphorylation of protein kinase B (AKT). Interestingly, aspalathin also improved mitochondrial respiration and function, which was evident by an increased expression of carnitine palmitoyltransferase 1 (*Cpt1*), fatty acid transport protein 1 (*Fatp1*), sirtuin 1 (*Sirt1*), nuclear respiratory factor 1 (*Nrf1*), and transcription factor A, mitochondrial (*Tfam*). Importantly, our results showed that aspalathin treatment was effective in ameliorating the devastating outcomes of insulin resistance and mitochondrial dysfunction that are linked with an undesired pro-inflammatory response, by reducing the levels of well-known pro-inflammatory markers such as interleukin-6 (*IL-6*), tumor necrosis factor-alpha (*TNF- α*), and protein kinase C-theta (PKC- θ). Thus, beyond improving glucose uptake and insulin signaling, the current study

brings a new perspective in the therapeutic benefits of aspalathin in improving mitochondrial respiration and blocking inflammation to attenuate the detrimental effect of palmitate in skeletal muscle cells.

Key words

Type 2 diabetes mellitus • Skeletal muscle insulin resistance • Aspalathin • Insulin signaling • Mitochondrial dysfunction and inflammation

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Introduction

The rapid increase in the prevalence of diabetes mellitus, particularly type 2 diabetes mellitus (T2DM) [1] is of major concern, due to its significant contribution to the overall increase in global deaths [2]. Modifiable risk factors such as excessive nutrient intake, which are usually coupled with a sedentary lifestyle, are acknowledged to be the main instigators in the development of diverse metabolic diseases including

T2DM [3]. In fact, excessive body fat accumulation in a state of obesity is responsible for the development of insulin resistance, a major characteristic feature of T2DM [3]. Indeed, impairments in insulin signaling, mainly accompanied by suppressed muscle glucose homeostasis or derangements in free fatty acid oxidation within the skeletal muscle are recognized as established pathological mechanisms unique to T2DM [4]. This also explains the significance of the skeletal muscle in maintaining essential physiological processes, especially its role in regulating energy metabolism, including the uptake and utilization of major substrates like glucose and free fatty acids [5].

Notably, well-known interventions like physical activity are acknowledged to alleviate some metabolic complications by improving skeletal muscle glucose utilization, or even by modulating molecular mechanisms involved in energy metabolism and insulin signaling [6,7]. Regarding the latter, prime examples include effective regulation of insulin-dependent mechanisms like the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway. Briefly, through binding to the insulin receptor (IR), insulin can directly stimulate PI3K, which leads to the activation of protein kinases such as AKT that promote glucose uptake within the skeletal muscle [8]. Interestingly, accumulative research has indicated that diverse pharmacological compounds, including established antidiabetic drugs like insulin and metformin, mainly function through activation of such molecular mechanisms to reverse insulin resistance or alleviate complications linked with T2DM [9-11].

Likewise, available research has indicated that herbal medicine has the potential to ameliorate metabolic abnormalities linked with insulin resistance by improving insulin sensitivity, in part through effective regulation of PI3K/AKT pathways [12-14]. Particular interest has also been placed on characterizing and determining the relative levels of bioactive compounds found in herbal medicines, as the major therapeutic effects of these plants are attributed to these exceptional compounds. In fact, due to its envisaged health capabilities, there has been a general interest in understanding the therapeutic potential of bioactive compounds found in rooibos (*Aspalathus linearis*) in ameliorating metabolic diseases and improving human health [15-19]. As such, our research group has progressively explored the ameliorative effects of aspalathin, the major dihydrochalcone unique to rooibos, against metabolic complications linked with insulin resistance and T2DM

in various preclinical models. Available evidence suggests that beyond controlling substrate metabolism, including regulating the utilization of glucose and free fatty acids [20-22], aspalathin or extracts rich in this bioactive compound can modulate essential cellular mechanisms involved in cell survival, such as the PI3K/AKT signaling, mitochondrial respiration, and even autophagy to hinder cellular damage in various experimental models of diabetes [23-28]. Notably, there is not much information that has been reported on the direct impact of aspalathin in salvaging palmitate-induced insulin resistance, mitochondrial dysfunction, or suppressing pro-inflammatory markers in cultured C2C12 myotubes. Thus, beyond enhancing our current understanding of the therapeutic effects of aspalathin, the current study completes a global perspective on the link between insulin resistance and mitochondrial dysfunction. Lastly, this study also demonstrated the potential protective effect of aspalathin against palmitate-induced inflammation, which is also known as risk factor of T2DM.

Materials and Methods

Reagents

Murine C2C12 skeletal muscle cells (Catalog No. CRL-1772) were obtained from the American Type Culture Collection (Manassas, VA, USA). Aspalathin ($\geq 98\%$ purity, batch SZ1-356-54) was acquired from High Force Research LTD (Durham, UK). Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's phosphate-buffered saline (DPBS, pH 7.4 with calcium and magnesium), penicillin-streptomycin and luminescent ATP kit were obtained from Lonza (Walkersville, MD, USA). Horse serum (HS) and fetal calf serum (FCS) were from Biochrom (Berlin, Germany), and PrestoBlue™ cell viability reagent was purchased from Invitrogen (Karlsruhe, Germany). 2-Deoxy- ^3H -D-glucose was from American Radiolabelled Chemicals (St Louis, MO, USA). Bradford protein assay kits were from Bio-Rad Laboratories (Hercules, CA, USA). Cell Signalling Technology (Beverly, MA, USA) supplied primary antibodies, including protein kinase C-theta (PKC- θ), p/PKC- θ (^{Ser643/676}), protein kinase B (AKT), p-AKT (Ser473) (^{Ser473}), as well as insulin receptor (IR). The reference control, beta (β)-actin, and the secondary antibodies; goat-anti-mouse and goat-anti rabbit IgG – horseradish peroxidase (HRP) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Quant-iT™

PicoGreen™ dsDNA quantitation assay kits, TRIzol reagent, Turbo DNase Kit, and all PCR probes were supplied by ThermoFisher Scientific, Inc. (Waltham, MA, USA). Taqman Master Mix, QIAzol, QuantiTect Reverse Transcription Kit were obtained from Qiagen (Valencia, CA, USA). Reagents for Seahorse experiments such as oligomycin, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), rotenone, Antimycin A were purchased from Agilent (Santa Clara, CA, USA). Palmitic acid (C18:0) and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), except when specified.

Cell culture treatments

An established experimental model was used to culture and differentiate skeletal (C2C12) muscle cells [29]. Briefly, cells were cultured and sub-cultured in DMEM containing 4.5 g/l glucose and 20 mM HEPES, supplemented with 10 % (v/v) FCS, 100 U/ml penicillin, and 100 µg/ml penicillin-streptomycin for 3 days or until 90-100 % confluent. Upon confluency, these myoblasts were differentiated by switching to low serum DMEM containing 2 % HS to induce myotube formation. Then, C2C12 myotubules were seeded into cell bind 24-well plates (25000 cells/well), 96-well plates (5000 cells/well) or 6-well plates (75000 cells/well); and for seahorse, 96 well XF-96 microplate plates (12000 cells/well) were used. Media was replaced every 48 h, on day 5 cells were fully differentiated into myotubules, and then further experiments were performed.

Induction of insulin resistance and treatment with aspalathin

Upon confluence, insulin resistance was induced by culturing C2C12 myotubules in DMEM containing 2 % BSA, 5.5 mM glucose and 0.75 mM palmitate for 16 h, as previously described [29]. On the other hand, experimental control cells were cultured in DMEM without palmitate. After induction of insulin resistance, cells were serum and glucose starved before treatment with aspalathin, which was prepared as previously described [21]. For treatment, the cells were exposed for 1 h in either 1 µM insulin (used as a positive control) or 10 µM aspalathin that was diluted in 2 % BSA DMEM culture media with or without palmitate. Choice of dose and treatment time for aspalathin was based on previously published research [21].

Determination of cell viability and metabolic activity

The PrestoBlue™ dye was used as a measure of cell viability, whilst the luminescent ATP kit was used to evaluate metabolic activity. Briefly, after treatment 10 µl PrestoBlue™ was added to each well and incubated for 40 min at room temperature and absorbance (570 nm) was measured after 10 min using PHERAstar® plate reader (BMG LABTECH, Germany). To measure the level of ATP within the cell, the luminescent ATP kit was used, as described by the manufacturer. ATP luminescence was read using a BioTek FLx800 plate reader and Gen 5 software for data acquisition (BioTek Instruments Inc., Winooski, VT, USA).

Determination of glucose uptake

Briefly, the glucose uptake test was performed using radiolabeled 2-Deoxy-[³H]-D-glucose, as previously described [20,21]. Briefly, after 16 h of incubation with palmitate and treatment with or without aspalathin for 1 h, 0.5 µCi/ml 2-Deoxy-[³H]-D-glucose was added to each well and incubated at 37 °C in 5 % CO₂ and humidified air for 15 min. Thereafter 2-Deoxy-[³H]-D-glucose as assessed by liquid scintillation using (2220 CA, Packard Tri-Carb series, PerkinElmer, Downers Grove, IL, USA).

mRNA expression analysis

Total RNA was extracted from treated C2C12 myotubules using Qiazol buffer according to the manufacturer's instructions, thereafter RNA concentration was determined using NanoDrop spectrophotometer (Thermo Scientific, USA). To remove contaminating genomic DNA from RNA, samples were DNase treated using QuantiTect Reverse Transcription Kit, and total RNA was reverse transcribed into cDNA according to the manufacturer's recommendations. Differential gene expression was analyzed by quantitative RT-PCR using QuantStudio™ 6 (Applied Biosystems, CA, USA). Gene expression, for interleukin (*IL*)-6, tumor necrosis factor alpha (*Tnf-α*), fatty acid transport protein 1 (*Fatp1*), carnitine palmitoyltransferase 1 (*Cpt1*), uncoupling protein 2 (*Ucp2*), NAD-dependent deacetylase sirtuin 1 (*Sirt1*), nuclear respiratory factor 1 (*Nrf1*) and transcription factor A, mitochondrial (*Tfam*) was calculated as delta Ct using Actin beta (Act b) for normalization and relative to the untreated (experimental) control group normalized to a value of 100 %, stated as "relative mRNA expression" in the y axis label. The list of gene expression probes used in this study as supplied in Table 1.

Table 1. The list of mRNA probes used in the study.

Probe	Role	Assay ID
Interleukin 6 (<i>IL-6</i>)	Inflammation	Mm00446190_m1
Tumor necrosis factor alpha (<i>Tnf-α</i>)	Inflammation	Mm00443258_m1
Fatty acid transport protein 1 (<i>Fatp1</i>)	Lipid metabolism	Mm00444340_m1
Carnitine palmitoyltransferase 1 (<i>Cpt1</i>)	Lipid metabolism	Mm00487191_g1
Uncoupling Protein 2 (<i>Ucp2</i>)	Mitochondrial bioenergetics	Mm00627599_m1
Sirtuin (<i>Sirt1</i>)	Mitochondrial biogenesis	Mm01168521_m1
Nuclear Respiratory Factor 1 (<i>Nrf1</i>)	Mitochondrial biogenesis	Mm01135606_m1
Transcription factor A, mitochondrial (<i>Tfam</i>)	Mitochondrial biogenesis	Mm00447485_m1
Actin beta (<i>Act b</i>)	Housekeeping	Mm02619580_g1

Cellular bioenergetics

An established protocol was used to assess cellular bioenergetics [22,30]. Briefly, before the measurements of cellular respiration, treated cells were removed from the CO₂ incubator and the medium was changed to XF Assay Medium containing 8 mM glucose with or without palmitate, aspalathin or etomoxir (1 mM), then equilibrated for another 1 h at 37 °C without CO₂. Thereafter, the 96-well XF microplates plate was transferred to a Seahorse XF96 extracellular flux analyzer (Agilent Technologies, Chicopee, MA 01022) and basal respiration/glycolysis values determined in three assay cycles (1 min mixing, 2 min waiting, 3 min measuring). Then, oligomycin (2 µg/ml) was injected in port A, followed by FCCP (2.5 µM) injection in port B, rotenone and antimycin A (2.5 µM) port C and 2-Deoxy-[³H]-D-glucose (100 µM) in port D. Results were normalized to DNA using PicoGreen® dsDNA quantitation assay kit according to manufacturer's recommendations.

Western blot analysis

Western blot analysis was performed using a standardized protocol, as previously described [20,29]. Briefly, proteins were extracted using RIPA buffer, then 40 µg of heat denatured proteins were separated on a 10 or 12 % SDS-PAGE gel and transferred to a PVDF-P membrane. Successful transfer was determined using Ponceau stain. Thereafter membranes were blocked with 5 % (w/v) low-fat milk powder in Tris-buffered saline with Tween 20 (TBST) at room temperature for 2 h. Membranes were then labelled overnight at 4 °C with the relevant primary antibody (PKC- θ , p/PKC- θ (Ser643/676), pAKT, tAKT, and IR) and HRP conjugated secondary antibodies were applied for 1.5 h the following day. Proteins were detected and quantified by chemiluminescence using a Chemidoc-XRS imager and

Quantity One 1-D software (Biorad Laboratories, Hercules, CA, USA), respectively. Here, β -actin was used as the reference control.

Statistical analysis

All results are expressed as \pm standard error of the mean (SEM) of three independent biological experiments. The significant difference between groups was determined using one-way of variance (ANOVA) followed by Tukey's *post hoc* test using GraphPad Prism version 8.0.1 (GraphPad Software Inc., San Diego, CA, USA). Results were considered significant at $p \leq 0.05$. For PCR results, comparisons between groups were performed using one-way multivariate ANOVA, followed by unpaired Student *t*-test, and a $p \leq 0.05$ was deemed as statistically significant.

Results

Aspalathin restored cell viability and ATP production in palmitate-induced insulin-resistant C2C12 myotubules

The effects of palmitate and aspalathin on cell viability and metabolic activity were investigated in differentiated C2C12 myotubules. The results showed that insulin has an ability to improve cell viability and ATP production in normal cells (not exposed to palmitate) ($p < 0.05$ and $p < 0.001$, respectively) (Fig. 1A, B). Interestingly, 0.75 mM palmitate significantly reduced cell viability ($p < 0.01$) and ATP production ($p < 0.001$) when compared to experimental (normal) controls (Fig. 1A, B). The addition of aspalathin, either as a monotherapy or in combination with insulin, significantly improved cell viability ($p < 0.001$ and $p < 0.01$, respectively) and ATP production ($p < 0.001$) in cells exposed to palmitate (Fig. 1A, B). However, insulin, as a positive control did not have

a significant effect in improving cell viability and ATP production in cells exposed to palmitate (Fig. 1A, B).

Aspalathin improved glucose uptake and the expression levels of proteins involved in insulin signaling in palmitate-induced insulin resistant C2C12 myotubules

Determining the levels of cellular glucose uptake, including the modulation of protein markers involved in insulin signaling like AKT/IR remains essential to evaluate the capability of treatment compounds to ameliorate insulin resistance. Insulin as comparative control, significantly improved glucose uptake ($p < 0.001$) and the phosphorylation of AKT in the normal cells ($p < 0.001$) (Fig. 2A, B, C). However, exposure of C2C12 myotubules to 0.75 mM palmitate significantly suppressed glucose uptake ($p < 0.01$), including protein expression levels of IR (not statistically significant) and p/AKT ($p < 0.001$) when compared to an experimental control (Fig. 2A, B, C). Nonetheless, treatment with aspalathin, as monotherapy or in combination with insulin, improved glucose uptake ($p < 0.001$), IR protein expression ($p < 0.01$ and $p < 0.001$), and the phosphorylation of AKT ($p < 0.001$) (Fig. 2A, B, C). Notably, insulin as a comparative control, showed similar results to aspalathin relevant to glucose uptake ($p < 0.01$) and p/AKT ($p < 0.001$) in palmitate exposed cells (Fig. 2).

Aspalathin improved gene expression levels of some molecular markers of fatty acid transport and inflammation in palmitate-induced insulin resistant C2C12 myotubules

Briefly, aberrant cellular fatty acid transportation, which may lead to the downstream detrimental effects of inflammation, remains an important aspect to determine, as it is also linked with the exacerbation of insulin resistance. Accordingly, exposure of differentiated C2C12 myotubules to 0.75 mM palmitate resulted in markedly increased mRNA expression levels of fatty acid transporters like *Fatp1* ($p < 0.001$) and *Cpt1* ($p < 0.001$), including prominent markers of inflammation such as *IL-6* ($p < 0.001$), *Tnf- α* ($p < 0.001$), and PKC- θ ($p < 0.001$) in comparison to the experimental control (Fig. 3A-E). Treatment with aspalathin, either as a monotherapy or in combination with insulin, showed comparative results in significantly reducing these fatty acid transport and inflammation makers. Notably, insulin as a positive control, did not significantly affect the mRNA expression levels of *Fatp1* and *Cpt1*, or pro-inflammatory markers (*IL-6*, *Tnf- α* and

PKC- θ) in comparison to palmitate only exposed cells (Fig. 3).

Aspalathin improved cellular bioenergetics in palmitate-induced insulin resistant C2C12 myotubules

To characterize the effect of aspalathin on the process of mitochondrial bioenergetics, we measured the OCR (Fig. 5A) and ECAR (Fig. 5B) in palmitate-induced insulin resistant C2C12 myotubules using Agilent Seahorse XF Technology. After induction of insulin resistance with palmitate for 16 h, muscle cells displayed significantly suppressed basal OCR ($p < 0.01$), ATP production ($p < 0.01$), maximal respiration ($p < 0.001$), and spare respiratory capacity ($p < 0.05$) (Fig. 4C-F). Treatment with aspalathin showed comparative results to its combination with insulin in improving all markers of mitochondrial bioenergetics (Fig. 4C-F). The use of insulin, as a comparative control, failed to show any effect (except for increasing maximal respiration, $p < 0.001$) in improving mitochondrial bioenergetics using the current experimental model of C2C12 myotubules exposed to elevated concentrations of palmitate.

Aspalathin improved the expression levels of genes involved in mitochondrial energetics in palmitate-induced insulin resistant C2C12 myotubules

We further investigated the effect of aspalathin on the expression levels of genes involved in mitochondrial energetics in palmitate induced C2C12 myotubules. Here, exposure of differentiated C2C12 myotubules to 0.75 mM palmitate significantly reduced the mRNA expression levels of *Ucp2* ($p < 0.001$), *Sirt1* ($p < 0.001$), *Nrf1* ($p < 0.001$), and *Tfam* ($p < 0.001$) (Fig. 5A-D). Interestingly, also consistent with Seahorse data (Fig. 4), treatment with aspalathin, either as a monotherapy or in combination with insulin, significantly improved the expression levels of all analyzed genes ($p < 0.001$) (Fig. 5A-D). The use of insulin, as a comparative control, showed some effect in enhancing the expression *Ucp2* ($p < 0.001$), *Sirt1* ($p < 0.001$), *Nrf1* ($p < 0.001$), and *Tfam* ($p < 0.001$) in comparison to palmitate control (Fig. 5).

Discussion

Pathological features, insulin resistance and mitochondrial dysfunction, are known hallmarks of T2DM, and are widely studied through the application of diverse preclinical models that mimic human disease [31-33].

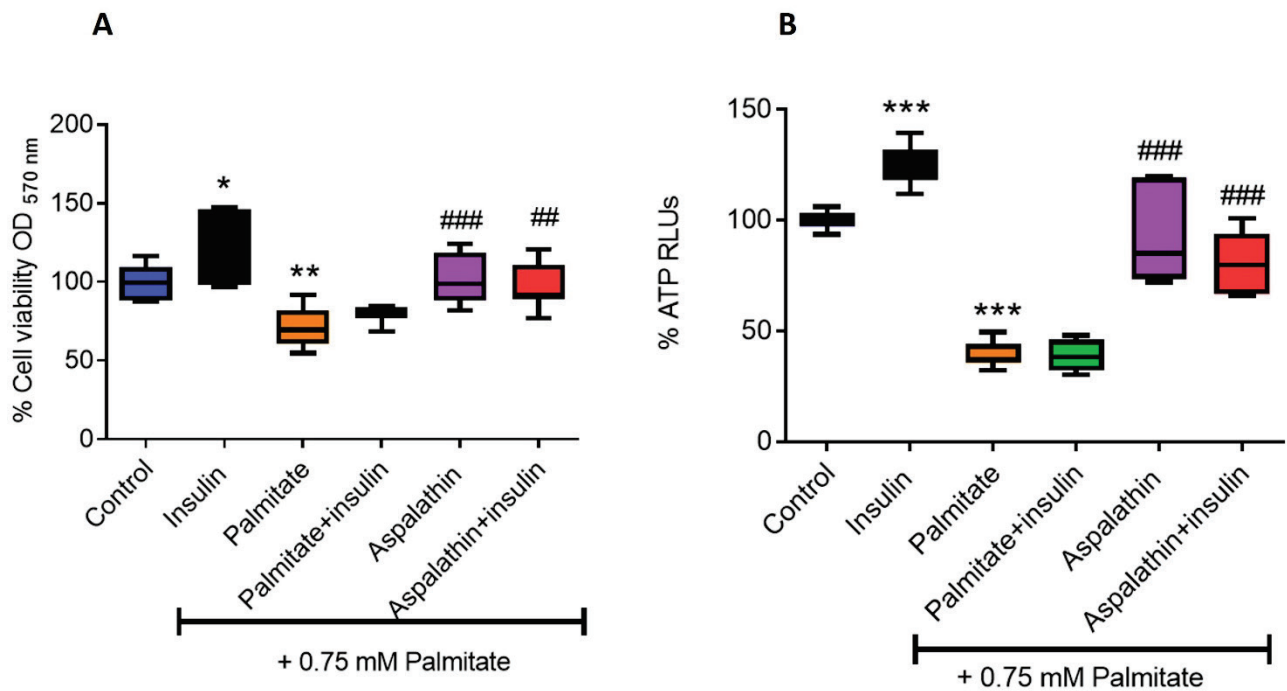


Fig 1. Effect of aspalathin on cell viability (**A**) and energy production (ATP) (**B**) in insulin resistant skeletal muscle (C2C12) cells. Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 μ M) for 1 h, while insulin (1 μ M) was added in the last 15 min as a positive control. As an endpoint measurement, cell viability was assessed by PrestoBlue dye and energy production (ATP) by luminescent ATP kit. Results are expressed as the mean of three independent experiments relative to the control at 100 % \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. experimental control: ## $p < 0.01$, ### $p < 0.001$ vs. palmitate control.

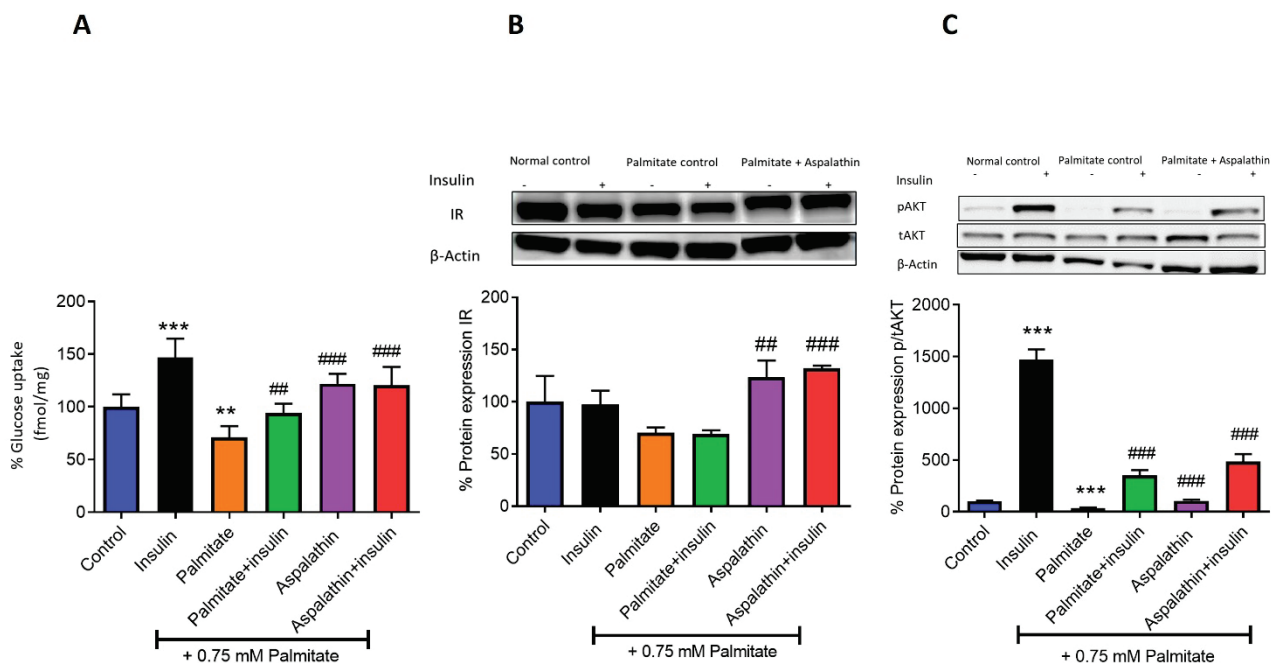


Fig. 2. Effect of aspalathin on glucose uptake (**A**), and protein expression levels of insulin receptor (IR; **B**), and phosphorylated protein kinase B (p/AKT; **C**) in insulin resistant skeletal muscle (C2C12) cells. Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 μ M) for 1 h, while insulin (1 μ M) was added in the last 15 min as a positive control. Treated cells were lysed and subjected to Western blot analysis. Results are expressed as the mean of three independent experiments relative to control set at 100 % \pm SEM. ** $p < 0.01$, *** $p < 0.001$ vs. experimental control: ## $p < 0.01$, ### $p < 0.001$ vs. palmitate control.

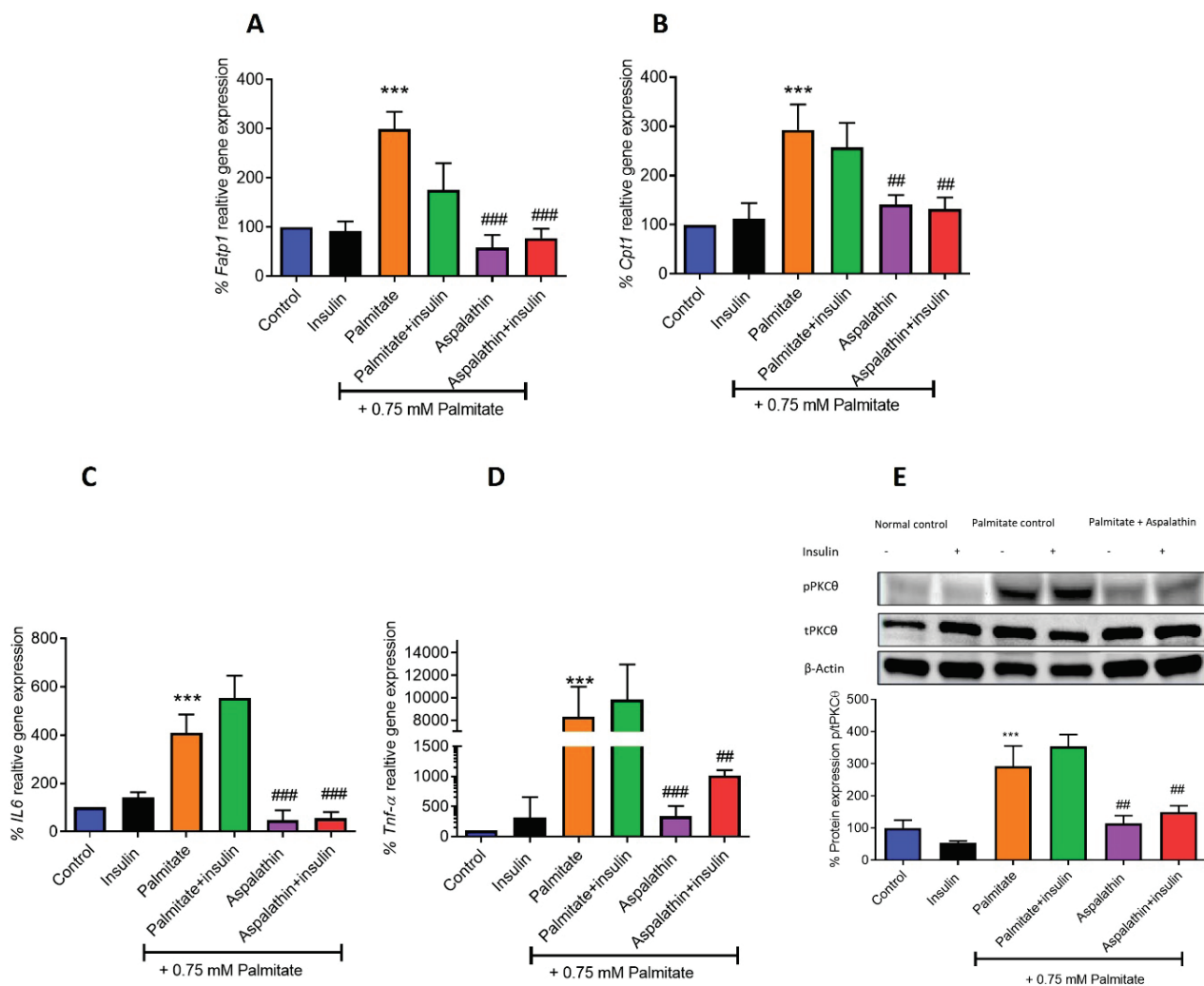


Fig. 3. Effect of aspalathin on the regulation of mRNA expression levels of genes involved in beta-oxidation like fatty acid transport protein 1 (Fatp1; **A**) carnitine palmitoyltransferase 1 (Cpt1; **B**), and genes involved in inflammation such as interleukin-6 (IL-6; **C**) and tumor necrosis factor alpha (TNF- α ; **D**), as well as protein expression of protein kinase C theta (PKC- θ ; **E**) in insulin resistant skeletal muscle (C2C12) cells. Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 μ M) for 1 h, while insulin (1 μ M) was added in the last 15 min as a positive control. Treated cells were lysed and quantified by RT-PCR and protein analysis. Results are expressed as the mean of three independent experiments relative to control set at 100 % \pm SEM. *** $p < 0.001$ vs. experimental control; ## $p < 0.01$, ### $p < 0.001$ vs. palmitate control.

Research from our group has explored an *in vitro* model of exposing cultured cells to elevated concentrations of palmitate as a feasible strategy to study pathological mechanisms of T2DM, including the development of insulin resistance and mitochondrial dysfunction [21,29,34,35]. In fact, there has been a growing interest in understanding the cause-effect relationship between insulin resistance and mitochondrial dysfunction during the pathogenesis of T2DM, especially within the skeletal muscle [31-33]. As such, beyond inducing alterations in substrate metabolism which may be characterized by suppressed glucose metabolism, exposing cultured cells to elevated concentrations of palmitate (0.75 mM) for 16 h has been shown to significantly hinder the process of mitochondrial respiration, including reducing the

expression of major genes involved insulin signaling and mitochondrial function [22,36]. Other studies have reported that oxidative stress, which is often exacerbated by mitochondrial dysfunction, might also accelerate cellular apoptosis, a consequence that has been explored in experimental models of metabolic disease [37,38]. Indeed, previous reviews have critically discussed the pathological role of apoptosis in the development of T2DM, especially its implication in the destruction of pancreatic β -islet cells [39,40]. Further, highlighting the importance of understanding the pathological features of T2DM, specifically mitochondrial dysfunction in order to protect against destructive processes involving apoptosis or accelerated cell death.

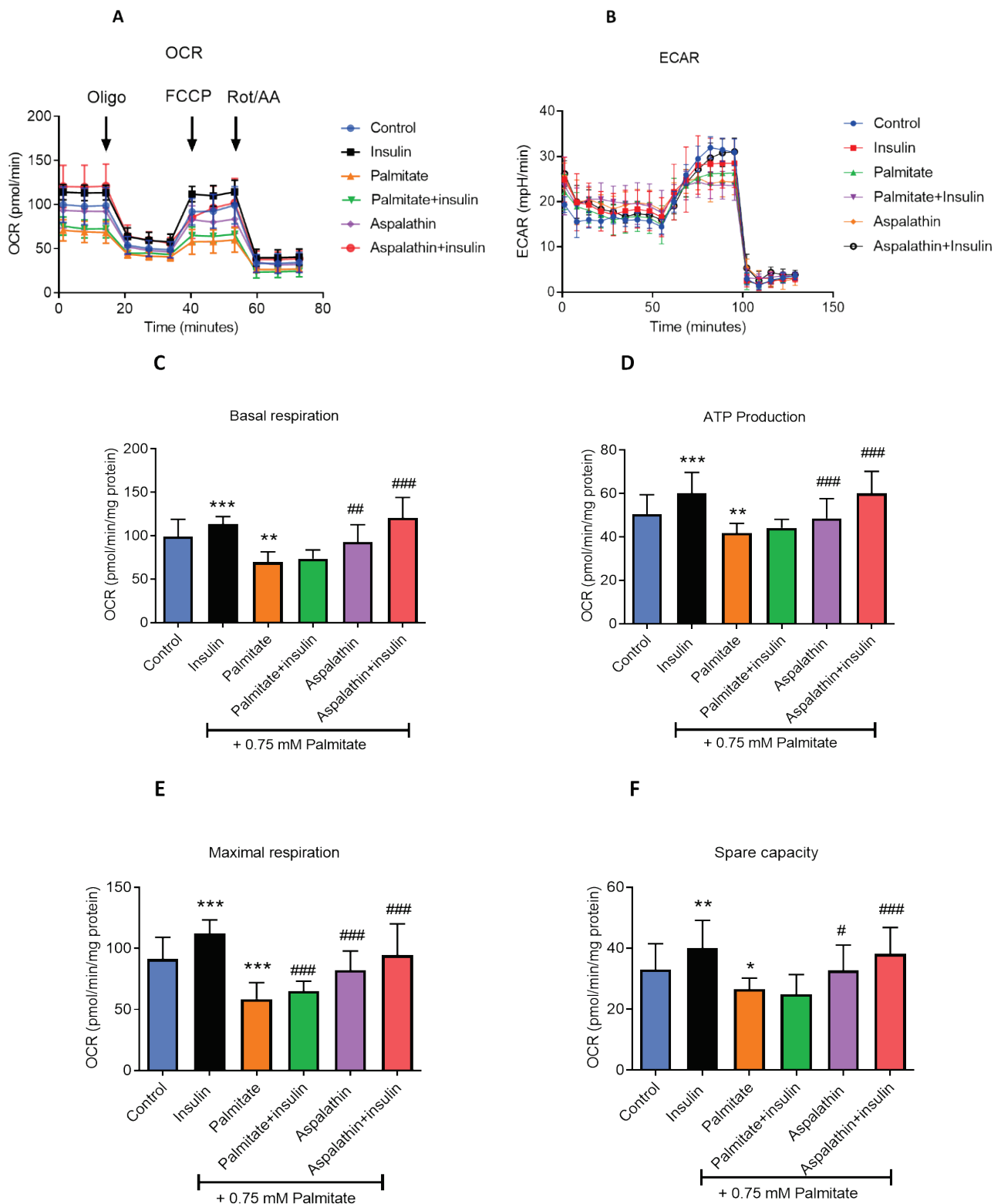


Fig. 4. Effect of aspalathin on the regulation of mitochondrial bioenergetics, including basal oxygen consumption rate (OCR; **A**) and extracellular acidification rates (ECAR; **B**) of all treatments, before independent analysis of basal OCR (**C**), adenosine triphosphate (ATP) production (**D**), maximal respiration (**E**), and spare respiratory capacity (**F**) in insulin resistant skeletal muscle (C2C12) cells. Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 1 h, thereafter cultured with aspalathin (10 μ M) for 1 h, while insulin (1 μ M) was added in the last 15 min as a positive control. The OCR and ECAR were determined using Seahorse XF-96 Metabolic Flux Analyzer. Groups (A, B, C, D, E and F) show data of changes in OCR, ECAR, in response to the sequential administration (arrows) of oligomycin (2 μ g/ml) port A, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (2.5 μ M) port B, antimycin and rotenone (2.5 μ M) port C. Results are expressed as mean \pm SEM of 3 independent experiments. * $p < 0.05$, ** $p < 0.001$ *** $p < 0.001$ vs. experimental control; # $p < 0.05$, ## $p < 0.05$, ### $p < 0.001$ vs. palmitate control.

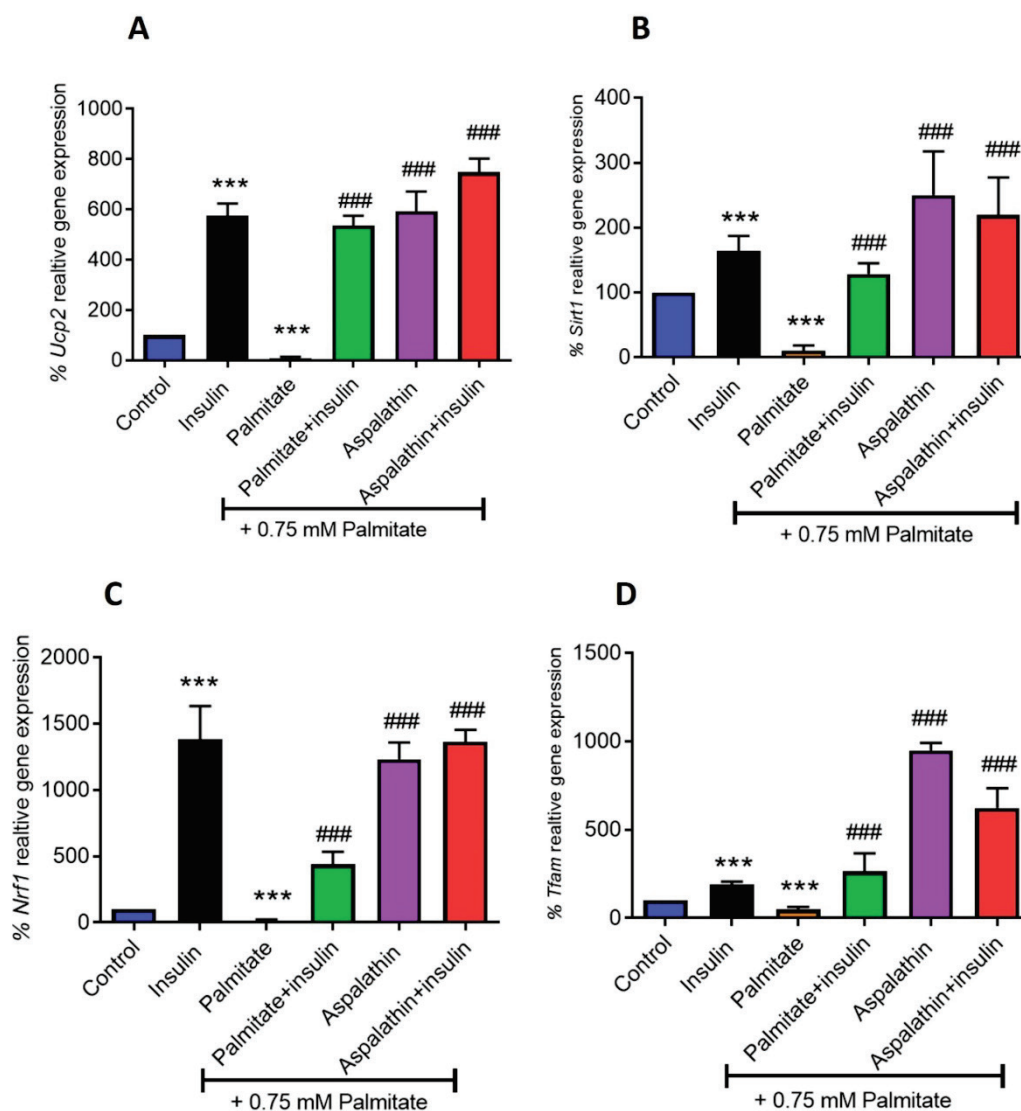


Fig. 5. Effect of aspalathin on the regulation of mRNA expression levels of genes involved mitochondrial function, including uncoupling protein 2 (Ucp2; **A**), silent mating type information regulation 2 homolog (Sirt1; **B**), nuclear respiratory factor 1 (Nrf1; **C**) and transcription factor A, mitochondrial (Tfam; **D**) in insulin resistant skeletal muscle (C2C12) cells. Briefly, C2C12 myotubules were treated with or without 0.75 mM palmitate for 16 h, thereafter cultured with aspalathin (10 μ M) for 1 h, while insulin (1 μ M) was added in the last 15 min as a positive control. Treated cells were lysed and quantified by RT-PCR analysis. Results are expressed as the mean of three independent experiments relative to control set at 100% \pm SEM. *** $p < 0.001$ vs. experimental control; ### $p < 0.001$ vs. palmitate control.

Notably, palmitate exposure, as an experimental model to mimic the toxic effects of lipid overload, has been increasingly studied [41]. In fact, the current study showed that exposing skeletal muscle (C2C12) myotubules to elevated concentrations of palmitate could effectively reduce glucose uptake and cell viability, and this was consistent with suppressing mitochondrial respiration, including genes involved in this process, such as *Ucp2*, *Sirt1*, *Nrf1*, and *Tfam*. Interestingly, to further characterize the classical features T2DM, the current study also showed that exposing C2C12 myotubules to elevated concentrations of palmitate resulted in enhanced levels of molecular markers, such as mRNA levels of

IL-6, *Tnf- α* , and phosphorylation of PKC- θ indicating an abnormal inflammatory response. This certainly affirms the relevance of using this *in vitro* model, of exposing C2C12 cells to elevated concentrations of palmitate, to assess the therapeutic effects of aspalathin against T2DM-related complications, including insulin resistance, mitochondrial dysfunction and an abnormal inflammatory response.

Intriguingly, research has progressively explored the use of plant-derived sources for their ameliorative effects against skeletal muscle insulin resistance, through the application of *in vitro* techniques. For example, the blueberry leaf extract was shown to attenuate

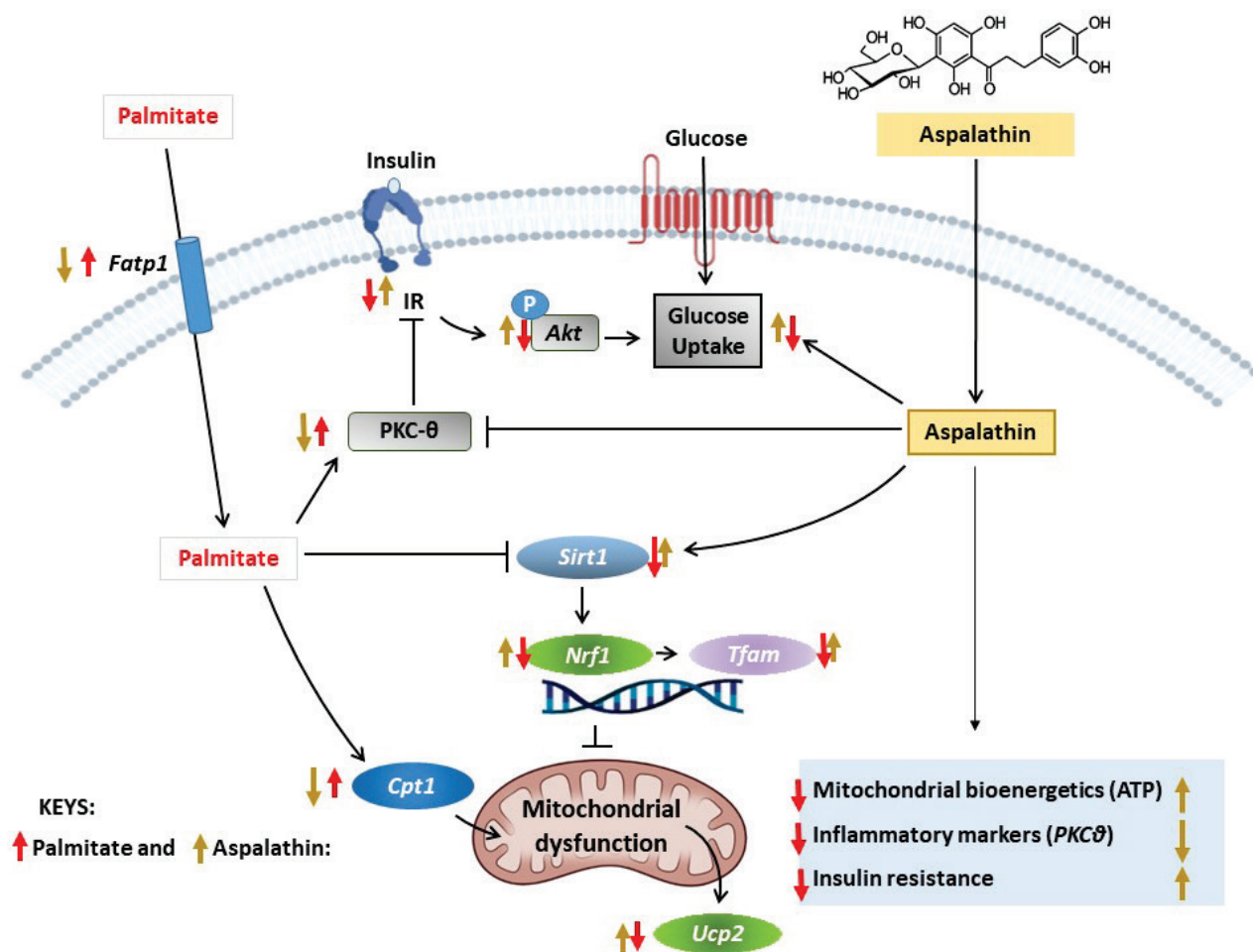


Fig. 6. An overview of therapeutic mechanisms by which aspalathin ameliorates palmitate-induced insulin resistance in skeletal muscle (C2C12) myotubules. Briefly, the major results in this study showed that aspalathin treatment could alleviate palmitate-induced skeletal muscle insulin resistance by enhancing glucose uptake and mitochondrial respiration blocking free fatty acid (FFA)-transport, as well as improving insulin signaling as partly demonstrated through increased expression insulin receptor (IR)/phosphorylation of protein kinase B (AKT) in cultured C2C12 myotubules. This bioactive compound could effectively attenuate inflammation by reducing the expression of markers such as interleukin-6 (*IL-6*), tumor necrosis factor- α (*TNF- α*), and protein kinase C- θ (*PKC- θ*). Moreover, markers of mitochondrial function included, included uncoupling protein 2 (*Ucp2*), NAD-dependent deacetylase sirtuin 1 (*Sirt1*), nuclear respiratory factor 1 (*Nrf1*) and transcription factor A, mitochondrial (*Tfam*).

TNF- α -induced insulin resistance by promoting glucose uptake and improving insulin signaling *via* upregulating AKT phosphorylation in cultured skeletal (C2C12) myotubules [42]. Alternatively, the well-known phytochemicals like quercetin, rutin and gallic acid, found in rooibos and other food sources, have been shown to hinder skeletal muscle atrophy and block cell apoptosis, in part by effectively scavenging for free radical species and improving mitochondrial function in cultured in C2C12 myotubules [43,44]. Here, the major results showed that aspalathin treatment could alleviate palmitate-induced skeletal muscle insulin resistance by enhancing glucose uptake and mitochondrial respiration, blocking FFA-transport, as well as improving insulin signaling as partly demonstrated through increased phosphorylation of AKT in cultured C2C12 myotubules

(Fig. 6). Interestingly, such findings are consistent with findings from other cellular models, mimicking complications of T2DM, reporting on the bioactive properties from rooibos, including aspalathin [22,35,36]. Furthermore, it has been reported that inflammation also plays a major role in the development of T2DM [45]. For example, macrophages and other immunocompetent cells can modulate an immune response and subsequently an inflammatory state through their secretion of cytokines such as TNF- α and IL-6, further taking part in different immune responses and protection against pathogens and diseases [46,47]. However, excessive immune activation, or exacerbated secretion of these pro-inflammatory cytokines has been implicated in the deterioration of metabolic complications linked with T2DM [46,48]. Similarly, there was increased mRNA expression of

TNF- α and *IL-6* upon the exposure of cells to palmitate in the current study. Such findings promote an important aspect entailing on the anti-inflammatory properties of this dihydrochalcone, as it effectively blocked PKC- θ phosphorylation in addition to reducing the mRNA levels of *IL-6* and *Tnf- α* in these palmitate-exposed skeletal muscle cells. In fact, this is validating the hypothesis that beyond its free radical scavenging properties [49], aspalathin presents with enhanced ameliorative effects against inflammation [50].

Figure 6: An overview of therapeutic mechanisms by which aspalathin ameliorates palmitate-induced insulin resistance in skeletal muscle (C2C12) myotubules. Briefly, the major results in this study showed that aspalathin treatment could alleviate palmitate-induced skeletal muscle insulin resistance by enhancing glucose uptake and mitochondrial respiration blocking free fatty acid (FFA)-transport, as well as improving insulin signaling as partly demonstrated through increased expression insulin receptor (IR)/phosphorylation of protein kinase B (AKT) in cultured C2C12 myotubules. This bioactive compound could effectively attenuate inflammation by reducing the expression of markers such as interleukin-6 (*IL-6*), tumor necrosis factor-alpha (*TNF- α*), and protein kinase C-theta (PKC- θ). Moreover, markers of mitochondrial function included, included uncoupling protein 2 (*Ucp2*), NAD-dependent deacetylase sirtuin 1 (*Sirt1*), nuclear respiratory factor 1 (*Nrf1*) and transcription factor A, mitochondrial (*Tfam*).

Conclusions

Through the use of an *in vitro* model of skeletal muscle insulin resistance, the current results support the notion that aspalathin presents with an enhanced therapeutic capacity to ameliorate diverse complications of metabolic syndrome, as previously reviewed [19]. Although such information remains relevant and could enhance the therapeutic development of aspalathin as a potential nutraceutical, the current study is not without limitations. Firstly, it remains essential to confirm these results using an established *in vivo* model of T2DM. Importantly, the *in vivo* model of T2DM could be used for a complete analysis of molecular mechanisms, linking both insulin resistance and mitochondrial dysfunction to better understand the therapeutic benefits of aspalathin and its potential synergetic effect with insulin (especially making use of *in vivo* models of T2DM). Notably,

although insulin can be used as an effective comparative control [36,51], other reference drugs, especially those specific for T2DM like metformin will add value to the understanding of therapeutic benefits of aspalathin in future studies. Lastly, future studies should also look at the therapeutic effects of aspalathin against oxidative stress-related cell death within the skeletal muscle, including the implications of inflammation during the development of T2DM.

Conflict of Interest

There is no conflict of interest.

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Abbreviations

AKT – protein kinase B, Cpt1 – carnitine palmitoyltransferase 1, ECAR – extracellular acidification rate, Fatp1 – fatty acid transport protein 1, FFA – free fatty acids, IL-6 – interleukin-6, IR – insulin receptor, Nrf1 – nuclear respiratory factor 1, OCR – mitochondrial oxygen consumption rate, PI3K – phosphoinositide 3-kinase, PKC- θ – protein kinase C-theta, Sirt1 – NAD-dependent deacetylase sirtuin 1, T2DM – Type 2 diabetes mellitus, Tfam – transcription factor A, TNF- α – tumor necrosis factor-alpha, Ucp2 – uncoupling protein 2.

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