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Isoorientin ameliorates lipid accumulation by regulating fat browning in palmitate-exposed 3T3-L1 adipocytes

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ABSTRACT

Stimulation of fat browning using natural bioactive products is regarded as one of the promising approaches to treat obesity and insulin resistance. Here, we investigated the physiological effects of isoorientin on glucose uptake and lipid accumulation in insulin resistant 3T3-L1 adipocytes. To achieve this, 3T3-L1 adipocytes were exposed to 0.75 mM palmitate for 24 h, to induce insulin resistance, before treatment with 10 μM isoorientin or the comparative controls such as CL-316,243 (10 μM), pioglitazone (10 μM) and compound C (1 μM) for 4 h. Relevant bioassays and Western blot analysis were conducted on these insulin resistant cells. Our results showed that palmitate exposure could induce insulin resistance and mitochondrial dysfunction as measured by reduction in glucose uptake and impaired mitochondrial bioenergetics parameters. However, treatment with isoorientin reversed these effects by improving glucose uptake, blocking lipid accumulation, and modulating the process of mitochondrial respiration. Mechanistically, isoorientin could mediate lipid metabolism by activating 5′ AMP-activated protein kinase (AMPK), while also effectively modulating the expression of genes involved in fat browning such as peroxisome proliferator-activated receptor gamma (PPAR)γ/α and uncoupling protein 1 (UCP1). In conclusion, isoorientin impacts insulin resistance in vitro by improving glucose uptake and mitochondrial function, consistent to modulating the expression of genes involved in energy metabolism and fat browning.

1. Introduction

Insulin resistance, which is characterized by decreased insulin-stimulated glucose uptake by insulin target cells remains the major underlying risk factor for the development of metabolic diseases such as type 2 diabetes (T2D) [1,2]. Besides insulin resistance, T2D is associated with obesity, a condition consistent with excessive lipid accumulation and the development of the metabolic syndrome [3]. Fascinatingly, the adipose tissue is well equipped to regulate energy homeostasis within the normal physiological state. In fact, two distinct types of fat cells are important for the regulation of essential metabolic processes in humans, these are white and brown adipocytes [4]. While white adipocytes are involved in the storage of excess energy as fat, the brown adipocytes are essential for thermogenic energy expenditure [5]. Consistently, fat browning is regarded as an ideal therapeutic approach for treating obesity and its associated insulin resistance [6].

Mechanistically, fat browning is associated with the activation of brown adipose tissue makers such as peroxisome proliferator-activated receptor alpha/gamma (PPARα/γ), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) and uncoupling protein 1 (UCP1), which plays an important role in maintaining beige adipocyte, modulating β-oxidation and

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Abbreviation

AMPK	5′ AMP-activated protein kinase
p-AMPK	phosphorylated 5′ AMP-activated protein kinase
ATP	adenosine triphosphate
BSA	bovine serum albumin
CO_2	carbon dioxide
CIDEA	cell death activator
COX8β	cytochrome c oxidase subunit 8 A
DEX	dexamethasone
DIO2	iodothyronine Deiodinase 2
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
FBS	fetal bovine serum
FFA	free fatty acid
IBMX	3-isobutyl-1-methylxanthine
LHX8	LIM Homeobox 8
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
ORO	oil red O
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K/Akt	phosphoinositide 3-kinase/protein kinase B
PPARα	peroxisome proliferator-activated receptor alpha
PPARγ	peroxisome proliferator-activated receptor gamma
PRDM16	PR domain containing 16
$NaHCO_3$	sodium bicarbonate
T2D	type 2 diabetes
TNF-α	tumour necrosis factor-alpha
TZs	thiazolidinediones
UCP 1	uncoupling protein 1
WHO	World Health Organisation

mitochondrial biogenesis [7,8]. As a result, fat browning has been therapeutically targeted with an aim to ameliorate obesity-associated complications. Some natural compounds, like resveratrol, have already been experimentally shown to significantly block fat accumulation and promote browning of white adipose tissue in high fat diet-fed mice [9]. Using 3T3-L1 adipocytes, our group and others have actively have screened different natural products for their potential in ameliorating palmitate-induced insulin resistance [10–12].

Preliminary findings have shown that isoorientin can ameliorate obesity-associated complications by suppressing adipogenesis in 3T3-L1 adipocytes [13]. Isoorientin is a *C*-glucosyl flavone commonly found in *Aspalathus linearis* (rooibos) and other medicinal plants [11,13,14]. Some in vitro evidence has shown that isoorientin can revert tumour necrosis factor-alpha induced insulin resistance in murine 3T3-F442A and human adipocytes [15]. However, although such in vitro evidence has been reported [13,15], a clear mechanism by which isoorientin ameliorates lipid accumulation or its impact on adipose tissue browning is unknown. In addition to assessing the beneficial effects of isoorientin on metabolic function, including improving glucose uptake and ameliorating lipid accumulation, the current study provides novel evidence on the impact of this natural compound on molecular mechanisms involved in fat browning in 3T3-L1 adipocytes exposed to palmitate.

2. Materials and methods

2.1. Reagents and kits

Isoorientin, CL-316,243 (CL), pioglitazone hydrochloride (purity 99%), palmitic acid (C18:0), dexamethasone (DEX), dimethyl sulfoxide (DMSO), 3-isobutyl-1-methylxanthine (IBMX), insulin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide) (MTT), Oil Red O (ORO), sodium bicarbonate ($NaHCO_3$), glycerol release kit, cell culture tested water as well as phenol red and glucose-free Dulbecco's modified Eagle's medium (DMEM) were obtained from Sigma-Aldrich® (St. Louis, MO, USA). Dulbecco's phosphate buffered saline (DPBS), DMEM, penicillin/streptomycin and trypsin were bought from Lonza Biowhitaker® (Walkersville, MD, USA). Fetal bovine serum (FBS) was purchased from Gibco®, Invitrogen™ (Gibco, Thermo Fisher, USA). 2-deoxy-[^{3}H]-D-glucose was purchased from American Radiolabelled Chemicals (St. Louis, MO, USA). Bradford and RC DC protein assay kits were obtained from Bio-Rad Laboratories (Hercules, CA, USA). The ViaLight™ plus adenosine triphosphate (ATP) kit was purchased from Lonza (Basel, Switzerland). Seahorse Microplates, DMEM assay media without phenol red for Seahorse XFe96, and Mito Stress assay test kits were obtained from Agilent (Santa Clare, CA, USA). 3T3-L1 mouse embryonic fibroblasts (ATCC Cat# CL-173TM) were obtained from the American Type Culture Collection (Manassas, VA, USA). Protease and phosphatase inhibitor tablets were purchased from Roche (South San Francisco, CA, USA). Cell Signalling Technology (Beverly, MA, USA) supplied primary antibodies including 5′ AMP-activated protein kinase (AMPK) (cat # 2532), phosphorylated 5′ AMP-activated protein kinase (p-AMPK) (Thr172) (cat # 2535S) and peroxisome proliferator-activated receptor gamma (PPARγ) (cat #2435), while Abcam (Cambridge, MA, USA) supplied UCP1 (cat # ab10983) and peroxisome proliferator-activated receptor alpha (PPARα) (cat # 24509). The housekeeping β-actin (cat # sc-47778) and secondary antibodies include goat anti-mouse (cat # sc-516102) and goat anti-rabbit horseradish peroxidase (IgG HRP) (cat # sc-2004) were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). All other chemicals were purchased from Sigma unless specified.

2.2. Cell culture and differentiation

The 3T3-L1 mouse embryonic fibroblasts were cultured in growth medium (DMEM supplemented with 10% FBS) at 37 °C in atmosphere of 5% CO_2 as previously described by [16]. Subsequently, the cells were sub-cultured every 2–3 days until they reached 80%–90% confluency. The cells were seeded into multi-well plate at density of 2×10^4 cells/mL which allowed them to reach confluency after 3 days of culture. Upon confluency (day 0), the pre-adipocytes were differentiated to mature adipocytes using a slightly modified method as previously described [12]. Briefly, growth media was substituted with adipocyte differentiation media (DMEM supplemented with 10% FBS, 0.5 mM IBMX, 1 μM DEX and 1 μg/mL insulin) for 2 days. On day 3, adipocyte differentiation media was changed to adipocyte maintenance media (DMEM containing 1 μg/mL insulin) for further 2 days. Thereafter, cells were cultured with growth medium that was refreshed after 2 days and kept in the same medium until day 8, before subsequent experiments.

2.3. Exposure of 3T3-L1 adipocytes to palmitate and preparation of compounds for cell culture

A previously described method was used to prepare palmitate used in the current experiments [11]. Briefly, fully differentiated 3T3-L1 adipocytes were exposed to growth media supplemented with 0.75 mM palmitate for 24 h at 37 °C in 5% CO_2 and humidified air to induce insulin resistance. Alternatively, all compounds including isoorientin, pioglitazone and CL-316,243 were dissolved in 100% DMSO to make stock solutions of 22.3, 25.5 and 10.73 mM, respectively. The stock solutions were further diluted in phenol red free DMEM (supplemented with 8 mM glucose, 3.7 g/L $NaHCO_3$, and 2% (w/v) BSA) to yield a final working solution with <0.001% DMSO, to avoid the toxic effect of DMSO as previously reported [17].

2.4. Treatment of insulin resistant 3T3-L1 adipocytes

Palmitate-induced insulin resistant 3T3-L1 adipocytes were serum and glucose starved for 30 min in DMEM without phenol red, serum or glucose to induce a phase of growth arrest, as previously described by [11]. Subsequently, the cells were treated for 4 h under standard tissue culture conditions in DMEM containing 8 mM glucose as a normal control; with or without palmitate (0.75 mM), compound C which is an AMPK inhibitor (10 μM), and positive controls such as CL-316,243 a selective β3-adrenergic agonist (1 μM), pioglitazone an antidiabetic-drug (10 μM) and isoorientin (10 μM). Thereafter, relevant assays were performed.

2.5. ATP and MTT assays

A modified MTT assay protocol, described by Mosmann in 1983 [18] was used to assess the metabolic activity of viable cells after treatment. Briefly, adipocytes were treated with MTT solution (2 mg/mL DPBS) and incubated for 30 min under standard tissue culture conditions. Additionally, ATP production was determined using fluorometric assay ViaLight™ plus ATP kit from Lonza (Basel, Switzerland), according to manufacturer's protocol. For the MTT assay, absorbance for purple formazan solution was read at 570 nm using BioTek®ELx 800 plate reader equipped with by Gen 5® software for data collection (BioTek Instruments Inc., Winooski, VT, USA). For ATP assay, the cells were incubated for a further 2 min at room temperature before reading the luminescence using a BioTek® FLx 800 plate reader equipped with Gen 5® software for data acquisition.

2.6. Glucose uptake assay

Glucose uptake was performed as previously described by Muller et al. [19], with some modifications. Briefly, treated adipocytes were incubated with glucose and phenol red free DMEM containing 0.5 μCi/ml 2-deoxy-[^{3}H]-D-glucose for 15 min under standard tissue culture parameters. Subsequently, 2-deoxy-[^{3}H]-D-glucose uptake was measured using liquid scintillation (2220 CA, Packard Tri-Carb series, PerkinElmer, Downers Grove, IL, USA). Glucose content (fmol/mg) was normalized with protein content which was quantified using Bradford assay read at 570 nm using BioTek® ELx800 plate reader supplied with Gen 5® software.

2.7. Oil Red O staining and glycerol release assay

Intracellular lipid accumulation was determined by measuring Oil Red O stain as previously described [20]. To quantify glycerol release, the culture media were collected and centrifuged to remove debris, and glycerol release was determined using fluorescent glycerol release assay kit from BioVision Inc. (Milpitas, CA, USA), following manufacturer's protocol. Subsequently, glycerol content was normalized to crystal violet stain measured at 570 nm using BioTek® ELx800 plate reader equipped with Gen 5® software.

2.8. Mitochondrial bioenergetics analysis

Mitochondrial respiration in insulin resistant 3T3-L1 adipocytes was assessed using XF96 Extracellular Flux Analyzer from Agilent as previously described [21], with slight modifications. In brief, 3T3-L1 pre-adipocytes were seeded in XF96 microplate plate at a density of 1×10^4 cells/well which reached confluency after 24 h. Subsequently, cells were differentiated and treated as previously described in section 2.2, and the Seahorse assay was performed on day 5 of differentiation. Subsequently, ATP production and maximal respiration was determined using Mito stress assay kit. After the assay, protein content was determined using Bradford protein assay, results were reported as oxygen consumption rate (OCR) (pmol O_2/min/mg protein).

2.9. Protein extraction and Western blot analysis

Proteins were extracted as previously described [11]. Western Blot analysis was done following the method described by [22]. Briefly, 30 μg and 40 μg of proteins were electrophoresed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated overnight at 4 °C with the following primary antibodies AMPK (1:1000), p-AMPK (Thr172) (1:800), PPARα (1:1000), PPARγ (1:800) and UCP1 (1:800). The β-actin (1:1000) was added as a loading control. The following day, the membranes were incubated with the relevant enzyme horseradish peroxidase conjugated secondary antibody (1:4000) in blocking buffer (5% skim milk) at room temperature for 90 min, and Clarity™ Western ECL Substrate (1:1 of luminal solution and reaction buffer) was added for 5 min. The proteins bands were detected using a ChemiDoc™ MP system (BioRad, Cressier, Switzerland) the signalling intensity of the protein bands was quantified using Image Lab software version 6.0.1.

2.10. Statistical analysis

Statistical calculations were performed using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test in GraphPad Prism software version 8.0.2 (GraphPad Software Inc., San Diego, CA, USA). Results are represented as the means ± SEM of three independent biological experiments. A p value of < 0.05 was considered as statistically significant.

3. Results

3.1. Isoorientin improved metabolic activity and glucose uptake in palmitate-exposed adipocytes

MTT assay was used to assess the effect of isoorientin in terms of mitochondrial activity in palmitate-induced insulin resistant 3T3-L adipocytes. Positive controls, CL-316,243 and pioglitazone had no effect in mitochondrial activity in normal cells, while the negative control compound C reduced mitochondrial activity by 25% ($p < 0.05$). Interestingly, treating these insulin resistant cells with isoorientin significantly enhanced mitochondrial activity by 33% ($p < 0.001$) relative to untreated palmitate control, this increase in MTT was more pronounced than those of positive controls CL-316,243 (28% $p < 0.001$) and pioglitazone (24% $p < 0.01$) (Fig. 1A). To confirm MTT results, ATP production was measured. Similarly, palmitate reduced ATP production by 23% ($p < 0.05$) compared to the normal control, and this reduction was normalized to that of

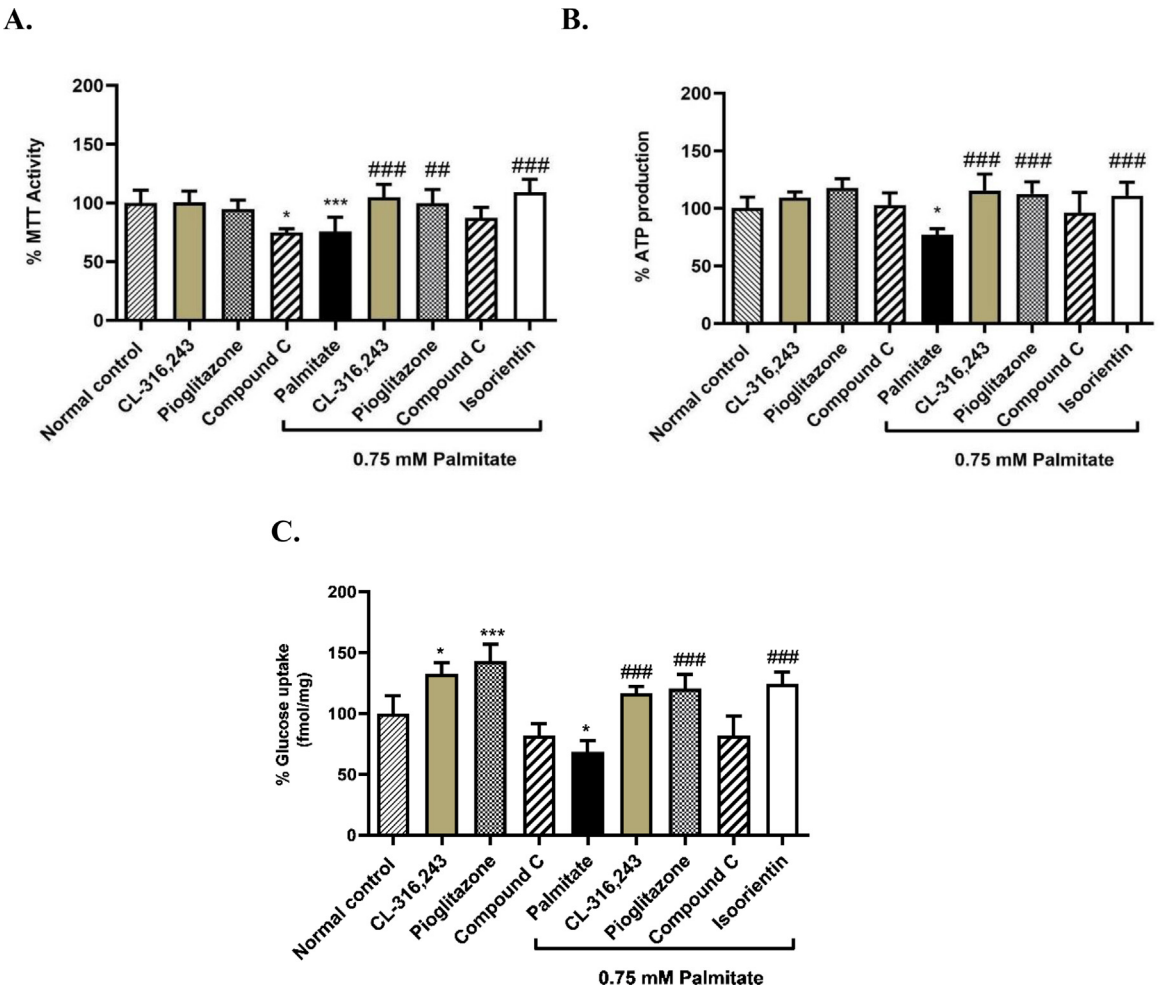

Fig. 1. Isoorientin improved mitochondrial activity as measured by MTT (A) and ATP production (B) assays, while also enhancing glucose uptake (C) in 3T3-L1 adipocytes exposed to 0.75 mM palmitate for 24 h. Cells were also treated with compound C, which was used as a 5′ AMP-activated protein kinase (AMPK) inhibitor (10 μM), positive controls such as CL-316,243 a selective β3-adrenergic agonist (1 μM), pioglitazone an antidiabetic-drug (10 μM) were also employed to compare the bioactivity of isoorientin. Results are expressed as mean ± SEM of three independent experiments. *p < 0.05, ***p ˂ 0.001 versus normal control and ## p ˂ 0.05, ###, p ˂ 0.001 versus palmitate control.

normal control by culturing with positive controls CL-316,243 (38% $p < 0.001$), pioglitazone (35% $p < 0.001$). Isoorientin on the other hand significantly improved ATP production by 34% ($p < 0.001$) (Fig. 1B).

Furthermore, glucose uptake in these insulin resistant 3T3-L1 adipocytes was assessed. The results showed both positive controls, CL-316,243 and pioglitazone increased glucose uptake by 32% and 43% ($p < 0.05$ and $p < 0.001$), respectively in comparison to untreated control (Fig. 1C). Palmitate significantly reduced glucose uptake by 32% ($p< 0.05$). Both positive controls CL-316,243 (48% $p < 0.001$) and pioglitazone (52% $p < 0.001$), including isoorientin 56% ($p < 0.001$) were able to enhance glucose uptake, when compared to the untreated palmitate control (Fig. 1C).

3.2. Isoorientin attenuated palmitate-induced lipid accumulation and enhanced lipolysis in palmitate-exposed adipocytes

The effect of isoorientin on lipid accumulation in palmitate-exposed adipocytes was assess using Oil Red O staining. The positive control CL-316,243 had no effect while, pioglitazone reduced lipid accumulation by 29% ($p < 0.01$) (Fig. 2A). Treatment with palmitate significantly increased lipid accumulation by 30% ($p < 0.01$) when compared to normal control. This increase was significantly reduced by both positive controls CL-316,243 43% ($p < 0.001$), pioglitazone 45% ($p < 0.001$), including isoorientin by 51% ($p < 0.001$) (Fig. 2A). In terms of glycerol release, palmitate did not have a significant impact on glycerol release. However, among treatments tested, only CL-316,243 demonstrated effect in enhancing glycerol release in these insulin resistant 3T3-L1 adipocytes (Fig. 2B).

3.3. Isoorientin improved palmitate altered mitochondrial bioenergetics in palmitate-exposed adipocytes

Mitochondrial bioenergetics was assessed to determine the effect of isoorientin on energy expenditure and mitochondrial dysfunction, as represented in Fig. 3A. The results showed that palmitate significantly reduced basal OCR by 38% ($p < 0.001$) relative to normal control, while isoorientin reverted this effect, increasing OCR by 36% ($p < 0.001$) when compared to the untreated palmitate control (Fig. 3B). Furthermore, ATP was measured after injecting 1 μM oligomycin, and results showed that compound C reduced ATP production by 57% ($p < 0.01$). Similarly, palmitate-exposure significantly reduced ATP production by 43% ($p < 0.001$) relative to normal control. Culturing these insulin resistant cells with isoorientin improved the ATP production by 117% ($p < 0.001$)

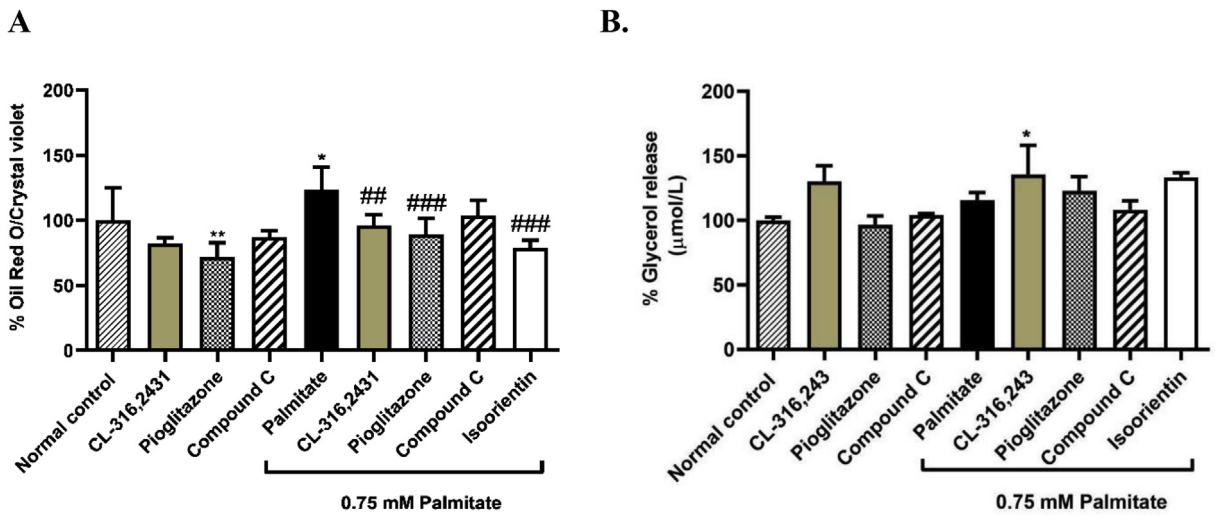

Fig. 2. Effect of isoorientin on lipid accumulation (A) and glycerol release (B) in 3T3-L1 adipocytes exposed to 0.75 mM palmitate for 24 h. Cells were also treated with compound C, which was used as a 5′ AMP-activated protein kinase (AMPK) inhibitor (10 μM), positive controls such as CL-316,243 a selective β3-adrenergic agonist (1 μM), pioglitazone an antidiabetic-drug (10 μM) were also employed to compare the bioactivity of isoorientin. Results are expressed as mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01 versus normal control and $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 versus palmitate control.

(Fig. 3C).

Maximal respiration was measured after injection with 7.5 μM carbonyl cyanide-4-trifluoromethoxy-phenylhydrazone (FCCP), and the results showed that the positive control CL-316,243 enhanced maximal respiration by 37%, when compared to the normal control (Fig. 3D). On the other hand, pioglitazone significantly improved maximal respiration after palmitate by 65% ($p < 0.001$), isoorientin treatment also enhanced OCR performance from 62% ($p < 0.001$), when compared to the untreated palmitate control (Fig. 3D).

In terms of spare respiratory capacity, the positive control CL-316,243 was able to increase respiratory capacity by 38% ($p < 0.001$), when compared to untreated palmitate-exposed cells (Fig. 3E). Interestingly, even after palmitate treatment, CL-316,243 was able to enhance maximal respiration by 44% ($p < 0.001$). Subsequently, isoorientin was able to significantly enhance spare respiratory capacity by 71% ($p < 0.001$), when compared to untreated palmitate control, and this increase was comparable to that of the positive control CL-316,243 (Fig. 3E).

3.4. Isoorientin enhanced the expression of genes involved in glucose and lipid homeostasis as well as fat browning in palmitate-exposed adipocytes

To partially understand the underlying mechanism by which isoorientin affects glucose uptake and lipid accumulation in insulin resistant adipocytes, we investigated the protein expression of AMPK, a master regulator of cellular energy homeostasis, and PPARγ, an essential mediator of adipogenesis. Here, our results showed that our positive controls CL-316,243 and pioglitazone were able to increase AMPK activation by 68% and 60% ($p < 0.05$) respectively (Fig. 4A). As expected, compound C reduced AMPK phosphorylation by 51% albeit not significant compared to normal control. Treatment with palmitate increased AMPK activation, however addition of isoorientin further increased the activation of AMPK by 30%, and this effect was significant ($p < 0.01$) when compared to the untreated control. This increase was comparable to both positive controls CL-316,243 and pioglitazone (Fig. 4A). Consistently, positive control CL-316,243 and isoorientin significantly increased the expression of PPARγ by 74% and 31% relative to normal and palmitate controls, respectively (Fig. 4B).; interestingly, although comparable to CL-316,243, isoorientin more effectively increased PPARy than pioglitazone, which is a PPARγ agonist.

We further tested the effect of isoorientin on the regulation of genes involved in fat browning, and these included PPARα and UCP1. The results showed that palmitate-exposure significantly downregulated PPARα protein expression by 45% ($p < 0.050$); while isoorientin ameliorated this effect by increasing PPARa exppression by 50% ($p < 0.05$) (Fig. 4C). Furthermore, isoorientin significantly increased UCP1 protein expression by 97% relative to the normal control, and 22% relative to untreated palmitate control (Fig. 4D). Additionally, exposure of untreated cells to palmitate enhanced UCP1 expression to the equivalent level of the positive control CL-316,243, which is a beta3-adrenergic agonist known to induce fat browning. Interestingly, isoorientin was more effective at stimulating UCP1 expression relative to all controls including CL-316,243 (Fig. 4D).

4. Discussion

An in vitro model of palmitate-induced insulin resistant 3T3-L1 adipocytes has been established to study obesity-associated complications, including insulin resistance [13,16,23]. Indeed, several studies have shown that concentrations of 0.2−0.75 mM palmitate are enough to induce insulin resistance in various cell lines over a 16−24 h period [11,24]. In our laboratory, we have explored metabolic alterations associated with palmitate-induced insulin resistance in 3T3-L1 adipocytes. As a prime example, we have shown that enhanced palmitate-exposure of 3T3-L1 adipocytes is associated with impaired glucose uptake and fatty acid oxidation [16]. These complications have been further demonstrated to be consistent with altered insulin signalling via the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway and dysregulated AMPK protein expression [12]. Although the effects on PI3K/AKT were not investigated, impaired glucose uptake in response to palmitate exposure in the current study was concomitant with enhanced lipid accumulation, reduced metabolic activity, and altered expression of genes involved in energy metabolism such as AMPK and PPARγ thus, inferring that palmitate-exposed 3T3-L1 adipocytes mimic obesity associated complications identified

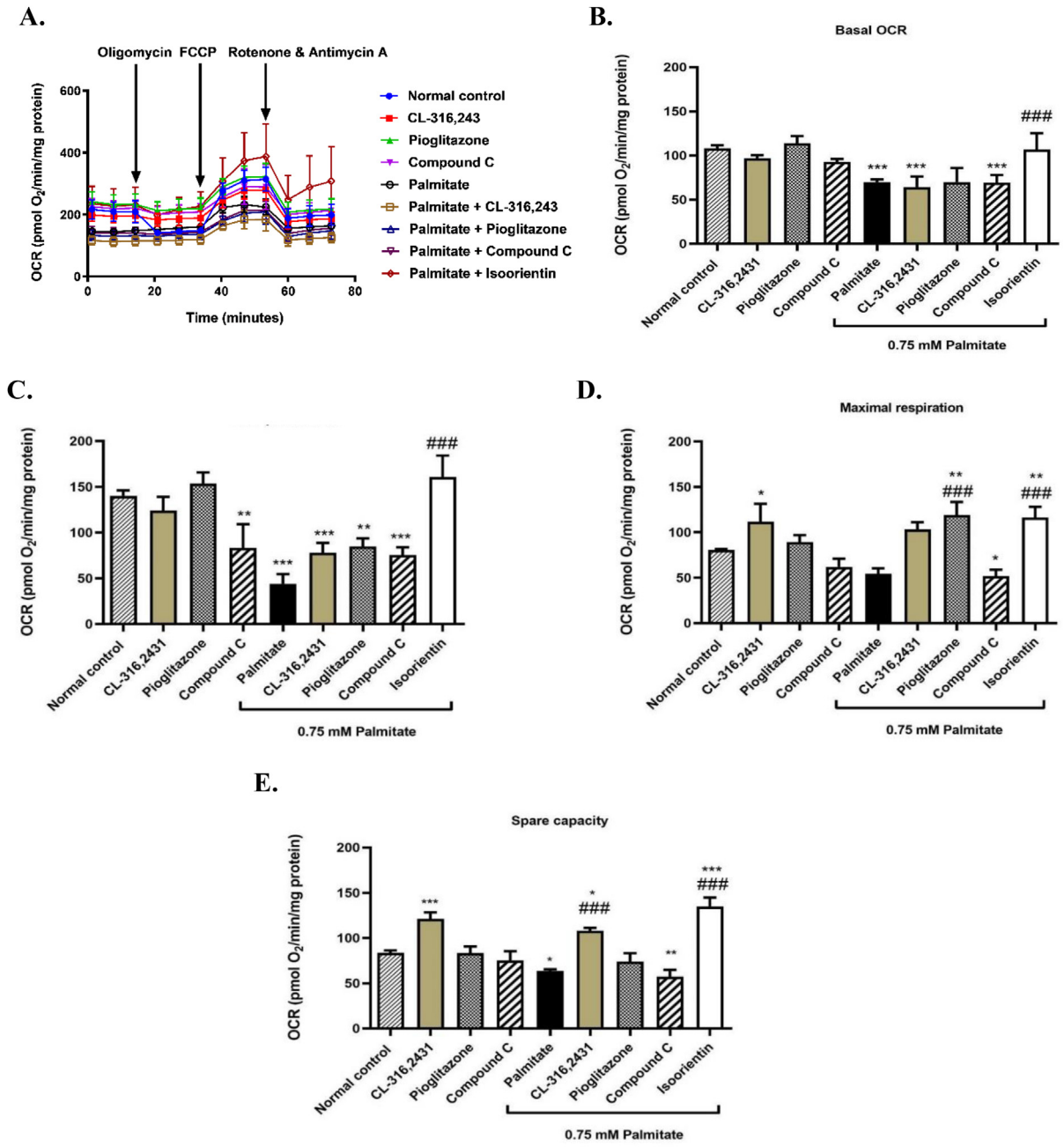

Fig. 3. Isoorientin improved mitochondrial bioenergetics (A), as shown by an increased in basal oxygen consumption rate (OCR) (B), ATP production (C), maximal respiration (D) and spare capacity (E) in 3T3-L1 adipocytes exposed to 0.75 mM palmitate for 24 h. Cells were also treated with compound C, which was used as a 5′ AMP-activated protein kinase (AMPK) inhibitor (10 μM), positive controls such as CL-316,243 a selective β3-adrenergic agonist (1 μM), pioglitazone an antidiabetic-drug (10 μM) were also employed to compare the bioactivity of isoorientin. Results are expressed as mean ± SEM of two independent experiments. *p < 0.05, **p ˂ 0.01, ***p ˂ 0.001 versus normal control and $^{###}$p ˂ 0.001 versus palmitate control.

in vivo [25].

A growing body of evidence shows that fat browning is a promising therapeutic strategy for combating human obesity and insulin resistance, because of its substantial energy-burning potential [26–28]. Activation of fat browning has been found to reverse obesity and insulin resistance in mice fed a high-fat diet [29]. In addition to their role in ameliorating insulin resistance, both AMPK and PPARγ have been reported to play essential role in stimulation and regulation of fat browning. For example, Wu and colleagues [30] showed that upon activation, AMPK can modulate fat browning by inducing the expression of brown fat thermogenic protein UCP1 in high-fat-diet fed mice. In agreement with these results, we found that isoorientin can activate AMPK consistent with interlinked regulation of PPARα/PPARγ/UCP1 in 3T3-L1 adipocytes exposed to palmitate. Recent evidence showed that elevated level of UCP1 is associated with enhanced glucose uptake and oxygen consumption in cultured adipocytes [31]. Similarly, in this study we found that isoorientin could improve glucose uptake in relation to increasing the expression of UCP1 and oxygen consumption rate in palmitate-induced 3T3-L1 adipocytes.

Furthermore, isoorientin treatment appeared to activate AMPK which was accompanied by reduced intracellular lipid

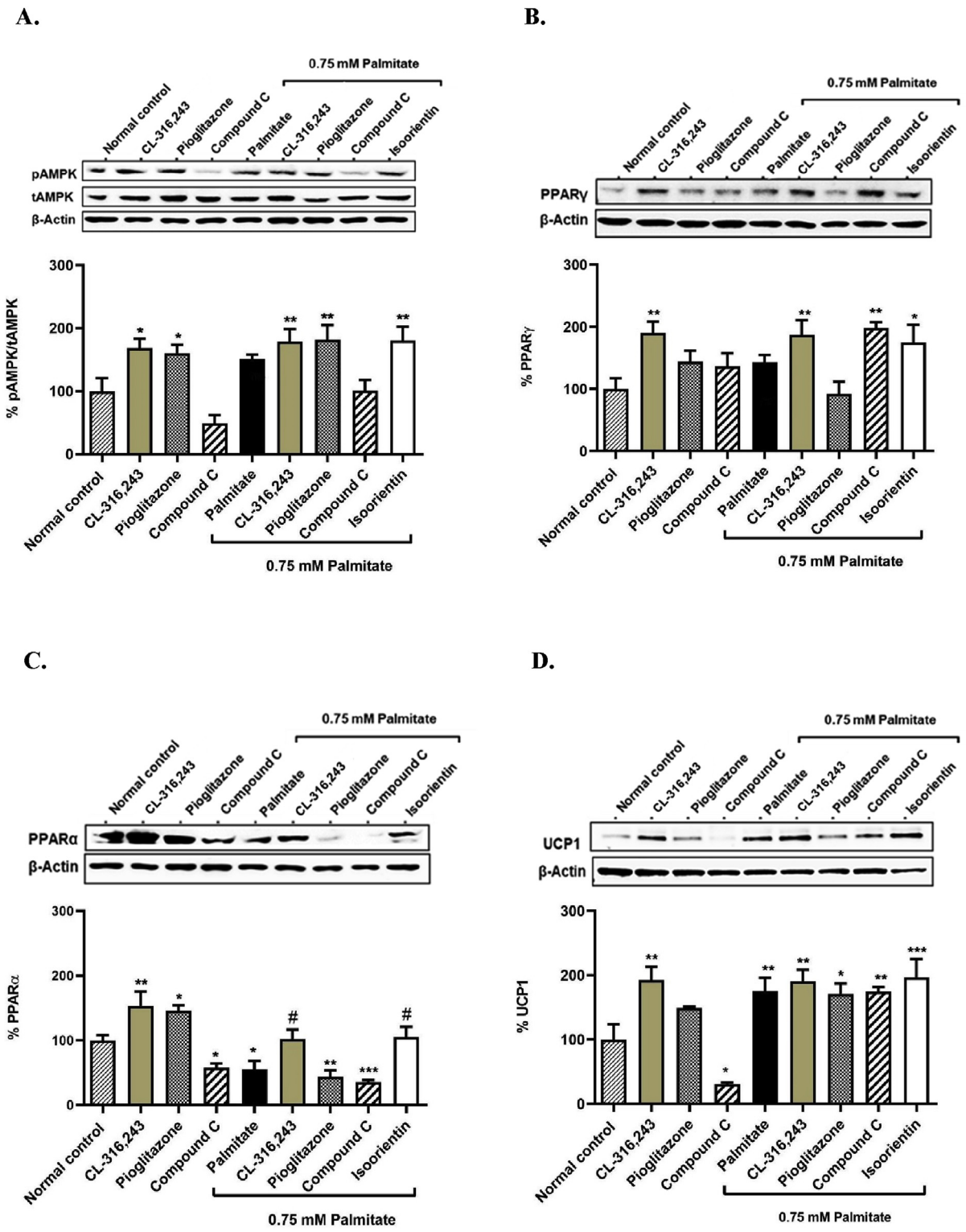

Fig. 4. Isoorientin improved pAMPK (A),PPARγ (B), PPARα (C), and UCP1 (D) protein expression in 3T3-L1 adipocytes exposed to 0.75 mM palmitate for 24 h. Cells were also treated with compound C, which was used as a 5′ AMP-activated protein kinase (AMPK) inhibitor (10 μM), positive controls such as CL-316,243 a selective β3-adrenergic agonist (1 μM), pioglitazone an antidiabetic-drug (10 μM) were also employed to compare the bioactivity of isoorientin. Results are expressed as mean ± SEM of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus normal control and $^{\#}p < 0.05$ versus palmitate control.

accumulation in these insulin resistant fat cells. These in vitro beneficial effects by isoorientin could well be linked to its activity in promoting fatty oxidation process, and thus translating directly to its potential to enhance the energy burning process, as illustrated elsewhere [32]. Certainly, we recently showed that, depending on the dose tested, isoorientin can improve metabolic activity and reduce lipid accumulation in differentiated adipocytes in part by promoting the phosphorylation of AKT and AMPK [33]. To support

this concept, in the current study, the results of increased energy expenditure were concomitant with enhanced mitochondrial respiration, with these effects even comparable with the positive controls used; further suggesting that isoorientin could play an important role in controlling mitochondrial energetics. However, although such putative benefits are observed with the use of isoorientin in ameliorating insulin resistance in vitro, the major challenge still persists as to whether the effective dose can be reached in vivo and how it can be selected for clinical practice. Hence, beyond the application of simple in vitro to in vivo extrapolation approaches for environmental compounds [34], this aspect is of importance to understand given the increasing interest on naturally-derived compounds and nutraceuticals for their role in combating metabolic diseases and related complications such as oxidative stress and inflammation [35–37]. In fact, food-derived products like rooibos are increasingly consumed for their envisaged health benefits [38], which have been shown to be related to its high phenolic content [39,40]. Although less is known regarding how isoorientin as a pure compound affects clinical parameters in obese subjects, consumption of 6 cups of rooibos tea for six weeks, which is known to contain relatively high levels of this flavone [41], has been linked with significantly improved lipid profile and redox status in adults at risk for developing cardiovascular disease [42]. Thus, in addition to testing its capability as a pure compound, other studies are necessary looking at the efficacy of food sources containing this flavone for their vast ameliorative effects against obesity in clinical settings.

Overall, the current findings provide essential information on how isoorientin impacts obesity-associated complications, however this preclinical model is not without limitations. Since we have shown that isoorientin can promote AMPK phosphorylation in conditions of insulin resistance, thus the combined use of this flavone with compound C should still be been tested. Precisely, additional experiments should focus on the combination use of isoorientin with compound c and its impact on basic metabolic assays such as glucose uptake, lipid accumulation, oxygen consumption, and UCP1 expression. Outstanding molecular work extends to understanding the modulatory effect of isoorientin on the expression of other markers involved in brown adipose tissue function such as PR domain containing 16 (PRDM16), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), LIM Homeobox 8 (LHX8), cell death activator (CIDEA), iodothyronine Deiodinase 2 (DIO2) and cytochrome c oxidase subunit 8A (COX8β). Also, investigations on the comparative effects of PPARα activators such as fenofibrate could add more information on the implicated mechanisms by which this dietary flavone ameliorates insulin resistance. This is especially vital since its known that fenofibrate can improve insulin sensitivity, reduce lipid consumption, and ameliorate other metabolic disease associated complications [43,44].

5. Conclusion and future perspectives

Our data support the impact of isoorientin on ameliorating obesity-related insulin resistance in vitro through improvement of glucose uptake, metabolic activity, mitochondrial energetics, as well as inhibition of lipid accumulation. Furthermore, partial speculation of mechanisms that might be implicated in this process are illustrated, as demonstrated by enhanced expression of AMPK/UCP1, and inhibition of PPARα following exposure of 3T3-L1 adipocytes to palmitate, all of which are molecules involved in energy metabolism. Indeed, further exploration of these processes and expansion of these results is necessary, especially using in vivo obesity-related experimental models.

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Data availability statement

All data used to support the findings of this study are included within the article. Raw data can be available on request after publication.

Declaration of competing interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

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References

[1] Taylor R. Insulin resistance and type 2 diabetes. Diabetes 2012;61(4):778–9.
[2] Nyambuya TMDP, Mxinwa V, Nkambule BB. Obesity-induced inflammation and insulin resistance: a mini-review on T-cells. Metabol Open 2019;3:100015. 2019.
[3] Sattar N, Gill JM. Type 2 diabetes as a disease of ectopic fat? BMC Med 2014;12:123.
[4] Ikeda K, Maretich P, Kajimura S. The common and distinct features of brown and beige adipocytes. Trends Endocrinol Metabol 2018;29(3):191–200.
[5] Park A, Kim WK, Bae KH. Distinction of white, beige and brown adipocytes derived from mesenchymal stem cells. World J Stem Cell 2014;6(1):33–42.
[6] Whittle AJ, Lopez M, Vidal-Puig A. Using brown adipose tissue to treat obesity - the central issue. Trends Mol Med 2011;17(8):405–11.
[7] Bargut TCL, Souza-Mello V, Aguila MB, Mandarim-de-Lacerda CA. Browning of white adipose tissue: lessons from experimental models. Horm Mol Biol Clin Invest 2017;31(1).
[8] Dludla PV, Nkambule BB, Tiano L, Louw J, Jastroch M, Mazibuko-Mbeje SE. Uncoupling proteins as a therapeutic target to protect the diabetic heart. Pharm Res 2018;137:11–24.
[9] Liao W, Yin X, Li Q, Zhang H, Liu Z, Zheng X, et al. Resveratrol-induced white adipose tissue browning in obese mice by remodeling fecal microbiota. Molecules (Basel, Switzerland) 2018;23(12).
[10] Tian X, Ye M, Cao Y, Wang C. Losartan improves palmitate-induced insulin resistance in 3T3-L1 adipocytes through upregulation of Src phosphorylation. Exp Clin Endocrinol Diabetes 2017;125(2):136–40.

[11] Mazibuko SE, Muller CJ, Joubert E, de Beer D, Johnson R, Opoku AR, et al. Amelioration of palmitate-induced insulin resistance in C2C12 muscle cells by rooibos (*Aspalathus linearis*). Phytomedicine 2013;20(10):813–9.
[12] Mazibuko-Mbeje SE, Dludla PV, Roux C, Johnson R, Ghoor S, Joubert E, et al. Aspalathin-enriched green rooibos extract reduces hepatic insulin resistance by modulating PI3K/AKT and AMPK pathways. Int J Mol Sci 2019;20(3).
[13] Poudel B, Nepali S, Xin M, Ki HH, Kim YH, Kim DK, et al. Flavonoids from Triticum aestivum inhibit adipogenesis in 3T3-L1 cells by upregulating the insig pathway. Mol Med Rep 2015;12(2):3139–45.
[14] Youn JY, Park HY, Cho KH. Anti-hyperglycemic activity of *Commelina communis* L.: inhibition of alpha-glucosidase. Diabetes Res Clin Pract 2004;66(Suppl 1):S149–55.
[15] Alonso-Castro AJ, Zapata-Bustos R, Gomez-Espinoza G, Salazar-Olivo LA. Isoorientin reverts TNF-alpha-induced insulin resistance in adipocytes activating the insulin signaling pathway. Endocrinology 2012;153(11):5222–30.
[16] Mazibuko SE, Joubert E, Johnson R, Louw J, Opoku AR, Muller CJ. Aspalathin improves glucose and lipid metabolism in 3T3-L1 adipocytes exposed to palmitate. Mol Nutr Food Res 2015;59(11):2199–208.
[17] Dludla PV, Jack B, Viraragavan A, Pheiffer C, Johnson R, Louw J, et al. A dose-dependent effect of dimethyl sulfoxide on lipid content, cell viability and oxidative stress in 3T3-L1 adipocytes. Toxicol Rep 2018;5:1014–20.
[18] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65(1–2):55–63.
[19] Muller CJ, Joubert E, de Beer D, Sanderson M, Malherbe CJ, Fey SJ, et al. Acute assessment of an aspalathin-enriched green rooibos (*Aspalathus linearis*) extract with hypoglycemic potential. Phytomedicine 2012;20(1):32–9.
[20] Sanderson M, Mazibuko SE, Joubert E, de Beer D, Johnson R, Pheiffer C, et al. Effects of fermented rooibos (*Aspalathus linearis*) on adipocyte differentiation. Phytomedicine 2014;21(2):109–17.
[21] Mazibuko-Mbeje SE, Dludla PV, Johnson R, Joubert E, Louw J, Ziqubu K, et al. Aspalathin, a natural product with the potential to reverse hepatic insulin resistance by improving energy metabolism and mitochondrial respiration. PloS One 2019;14(5):e0216172.
[22] Johnson R, Dludla P, Joubert E, February F, Mazibuko S, Ghoor S, et al. Aspalathin, a dihydrochalcone C-glucoside, protects H9c2 cardiomyocytes against high glucose induced shifts in substrate preference and apoptosis. Mol Nutr Food Res 2016;60(4):922–34.
[23] Hunnicutt JW, Hardy RW, Williford J, McDonald JM. Saturated fatty acid-induced insulin resistance in rat adipocytes. Diabetes 1994;43(4):540–5.
[24] Pinel A, Rigaudiere JP, Jouve C, Capel F. Modulation of insulin resistance and the adipocyte-skeletal muscle cell cross-talk by LCn-3PUFA. Int J Mol Sci 2018;19(9).
[25] den Besten G, Bleeker A, Gerding A, van Eunen K, Havinga R, van Dijk TH, et al. Short-chain fatty acids protect against high-fat diet-induced obesity via a PPARgamma-dependent switch from lipogenesis to fat oxidation. Diabetes 2015;64(7):2398–408.
[26] Cheng CF, Ku HC, Cheng JJ, Chao SW, Li HF, Lai PF, et al. Adipocyte browning and resistance to obesity in mice is induced by expression of ATF3. Commun Biol 2019;2:389.
[27] Zhang F, Ai W, Hu X, Meng Y, Yuan C, Su H, et al. Phytol stimulates the browning of white adipocytes through the activation of AMP-activated protein kinase (AMPK) alpha in mice fed high-fat diet. Food Funct 2018;9(4):2043–50.
[28] Thyagarajan B, Foster MT. Beiging of white adipose tissue as a therapeutic strategy for weight loss in humans. Horm Mol Biol Clin Invest 2017;31(2).
[29] Xu L, Nagata N, Chen G, Nagashimada M, Zhuge F, Ni Y, et al. Empagliflozin reverses obesity and insulin resistance through fat browning and alternative macrophage activation in mice fed a high-fat diet. BMJ Open Diabetes Res Care 2019;7(1):e000783.
[30] Wu L, Zhang L, Li B, Jiang H, Duan Y, Xie Z, et al. AMP-Activated protein kinase (AMPK) regulates energy metabolism through modulating thermogenesis in adipose tissue. Front Physiol 2018;9:122.
[31] Tews D, Pula T, Funcke JB, Jastroch M, Keuper M, Debatin KM, et al. Elevated UCP1 levels are sufficient to improve glucose uptake in human white adipocytes. Redox Biol 2019;26:101286.
[32] Lee WH, Kim SG. AMPK-Dependent metabolic regulation by PPAR agonists. PPAR Res 2010;2010.
[33] Ziqubu K, Muller CJF, Dludla PV, Mthembu SXH, Obonye N, Louw J, et al. Impact of isoorientin on metabolic activity and lipid accumulation in differentiated adipocytes. Molecules 2020;25:1773.
[34] Yoon M, Efremenko A, Blaauboer BJ, Clewell HJ. Evaluation of simple in vitro to in vivo extrapolation approaches for environmental compounds. Toxicol In Vitro 2014;28(2):164–70.
[35] Gurău F, Baldoni S, Prattichizzo F, Espinosa E, Amenta F, Procopio AD, et al. Anti-senescence compounds: a potential nutraceutical approach to healthy aging. Ageing Res Rev 2018;46:14–31.
[36] Olivieri F, Prattichizzo F, Grillari J, Balistreri CR. Cellular senescence and inflammaging in age-related diseases. Mediat Inflamm 2018:9076485.
[37] Patti AM, Al-Rasadi K, Giglio RV, Nikolic D, Mannina C, Castellino G, Chianetta R, et al. Natural approaches in metabolic syndrome management. Arch Med Sci 2018;14(2):422–41.
[38] Joubert E, de Beer D. Rooibos (*Aspalathus linearis*) beyond the farm gate: from herbal tea to potential phytopharmaceutical. South Afr J Bot 2011;77(4):869–86.
[39] Breiter T, Laue C, Kressel G, Gröll S, Engelhardt UH, Hahn A. Bioavailability and antioxidant potential of rooibos flavonoids in humans following the consumption of different rooibos formulations. Food Chem 2011;128(2):338–47.
[40] Dludla PV, Joubert E, Muller CJF, Louw J, Johnson R. Hyperglycemia-induced oxidative stress and heart disease-cardioprotective effects of rooibos flavonoids and phenylpyruvic acid-2-*O*-β-D-glucoside. Nutr Metab (Lond) 2017;14:45.
[41] Muller CJF, Malherbe CJ, Chellan N, Yagasaki K, Miura Y, Joubert E. Potential of rooibos, its major C-glucosyl flavonoids, and Z-2-(β-D-glucopyranosyloxy)-3-phenylpropenoic acid in prevention of metabolic syndrome. Crit Rev Food Sci Nutr 2018;58(2):227–46.
[42] Marnewick JL, Rautenbach F, Venter I, Neethling H, Blackhurst DM, Wolmarans P, et al. Effects of rooibos (*Aspalathus linearis*) on oxidative stress and biochemical parameters in adults at risk for cardiovascular disease. J Ethnopharmacol 2011;133(1):46–52.
[43] Koh KK, Han SH, Quon MJ, Yeal Ahn J, Shin EK. Beneficial effects of fenofibrate to improve endothelial dysfunction and raise adiponectin levels in patients with primary hypertriglyceridemia. Diabetes Care 2005;28(6):1419–24.
[44] Rachid TL, Penna-de-Carvalho A, Bringhenti I, Aguila MB, Mandarim-de-Lacerda CA, Souza-Mello V. Fenofibrate (PPARalpha agonist) induces beige cell formation in subcutaneous white adipose tissue from diet-induced male obese mice. Mol Cell Endocrinol 2015;402:86–94.