



Mifepristone enhances insulin-stimulated Akt phosphorylation and glucose uptake in skeletal muscle cells



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ABSTRACT

Mifepristone is the only FDA-approved drug for glycaemia control in patients with Cushing's syndrome and type 2 diabetes. Mifepristone also has beneficial effects in animal models of diabetes and patients with antipsychotic treatment-induced obesity. However, the mechanisms through which Mifepristone produces its beneficial effects are not completely elucidated.

Purpose: To determine the effects of mifepristone on insulin-stimulated glucose uptake on a model of L6 rat-derived skeletal muscle cells.

Results: Mifepristone enhanced insulin-dependent glucose uptake, GLUT4 translocation to the plasma membrane and Akt Ser⁴⁷³ phosphorylation in L6 myotubes. In addition, mifepristone reduced oxygen consumption and ATP levels and increased AMPK Thr¹⁷² phosphorylation. The knockdown of AMPK prevented the effects of mifepristone on insulin response.

Conclusions: Mifepristone enhanced insulin-stimulated glucose uptake through a mechanism that involves a decrease in mitochondrial function and AMPK activation in skeletal muscle cells.

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1. Introduction

Mifepristone, also known as RU486, is an antiprogestin that was primarily used as an emergency contraceptive or abortion-inducing drug because of its potent antagonist activity over progesterone receptor (Sun et al., 2014). Other studies have shown that mifepristone has potential antitumor antineoplastic effects, thus it has been used for the treatment of several types of cancer (Liu et al., 2016; Lu et al., 2016). Moreover, mifepristone is the only drug available with anti-glucocorticoid properties (Chen et al., 2014).

Glucocorticoids (GCs) are steroid hormones that regulate

numerous physiological processes that are responsible for the maintenance of organism homeostasis (Oakley and Cidlowski, 2011). Chronic exposition to GC is associated with adverse effects such as hyperglycemia, weight gain, hypertension, type 2 diabetes, muscle weakness, osteoporosis, depression and decreased immune function (Ramamoorthy and Cidlowski, 2013). Due to the effects of GCs in glucose homeostasis, several researchers have proved the effects of mifepristone in animal models of obesity and diabetes. One of the primary studies in the area showed that mifepristone improved muscle insulin resistance in rats fed with a high fat diet (Kusunoki et al., 1995). In differentiated 3T3-L1 adipocytes, mifepristone promotes adiponectin secretion through PPAR γ and enhances insulin-dependent glucose uptake, improving insulin sensitivity in high fat diet-induced obese animals (Hashimoto et al., 2013). In addition, mifepristone ameliorates diabetes symptoms in ob/ob mice (Gettys et al., 1997). Interestingly, in a knockout mouse

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for the macrophage migration inhibitory factor which developed glucose intolerance, treatment with mifepristone improved glucose tolerance, increased insulin secretion and enhanced insulin-triggered Akt Ser⁴⁷³ phosphorylation in the liver (Nikolic et al., 2013). Recently in high fructose fed-mice, mifepristone decreased lipid abnormalities and reduced insulin resistance (Priyadarshini and Anuradha, 2017). Many of these studies point to GCs as a part of the pathophysiology of metabolic disorders, however most of these conclusions are merely deduced by the effects of mifepristone.

In 2012, the FDA (Food and Drug Administration) approved the use of mifepristone for the treatment of elevated blood sugar levels in patients with Cushing's syndrome and type 2 diabetes mellitus (Morgan and Laufgraben, 2013; Rose and Herzig, 2013). Cushing's syndrome is a state in which an excess of circulating GCs exists in the organism, producing several metabolic disorders like hypertension, hyperglycemia, hyperinsulinemia, insulin resistance, type 2 diabetes, central obesity, fatigue, and depression among others (Hatipoglu, 2012; Morgan and Laufgraben, 2013). One of the most affected organs of this syndrome is the skeletal muscle (Kuo et al., 2013), in which about 80% of the glucose uptake is carried out and used in postprandial state (Otto and Dworzecki, 2002). Additionally, circulating GCs excess may induce protein degradation, which in turn leads to skeletal muscle atrophy (Kuo et al., 2013). Over-activation of GCs signaling in skeletal muscle may partially explain the glucose metabolism dysfunction in insulin resistance and diabetes (Rose and Herzig, 2013). It has been reported that treatment with mifepristone reduces hyperglycemia, insulin resistance and obesity (Rose and Herzig, 2013). Moreover, it has been reported that mifepristone ameliorates obesity and metabolic perturbations in patients with antipsychotic medication (Gross et al., 2009, 2010).

There is evidence that mifepristone could be useful in the management of glucose homeostasis perturbations in different contexts. However, the mechanisms by which mifepristone produces its effects are not completely understood, thus the purpose of this study is to determine the effects of mifepristone on insulin-dependent glucose uptake in L6 rat skeletal muscle cells in a non-GCs stimulate system.

2. Materials and methods

2.1. Cell culture

Wild type L6 cells and L6 cells stably expressing GLUT4 with a *myc* epitope in the first extracellular loop (L6-GLUT4*myc*) were cultured in alpha-MEM supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin-amphotericin B solution in a humidified atmosphere with 5% CO₂ at 37 °C. For differentiation to myotubes, cells were incubated in alpha-MEM supplemented with 2% (v/v) FBS for 5–7 days. L6-GLUT4*myc* were kindly donated by Dr. Amira Klip (Program for Cell Biology, Hospital for Sick Children, Toronto, ON, Canada).

2.2. Treatments

24 h before treatments, cells were washed with PBS and cultured in alpha-MEM supplemented with 2% dialyzed FBS for the remaining experimental time. Cells were then incubated with 10 μM mifepristone for the indicated times. For assessment of insulin signaling, a stimulus of 100 nM insulin was added in the last 30 min of mifepristone treatment.

2.3. Immunoblotting

Cells were washed with cold PBS and lysed using NP40. Lysates were centrifuged at 10 000 rpm for 10 min at 4 °C. Proteins were separated on SDS-polyacrylamide by molecular weight and transferred to PVDF membranes. Transferred membranes were blocked with 5% low-fat milk (Svelty, Nestlé) in TBS-T. Membranes were incubated overnight at 4 °C in primary antibodies: Anti-pP70S6K (Cell Signaling, Cat#9205), anti-p70S6K (Cell Signaling, Cat#2708), anti-pAkt (Cell Signaling, Cat#9271), anti-Akt (Cell Signaling, Cat #9272), anti-pAMPKα (Cell Signaling, Cat#2531), anti-AMPKα (Cell Signaling, Cat#2532), anti-Glucocorticoid receptor (Cell Signaling, Cat#3660) and anti-GAPDH (Sigma, Cat#G9545). After washing with TBS/0.1% Tween-20, membranes were incubated with secondary polyclonal antibodies anti-mouse (Cat #402335) and anti-rabbit (Cat #401315) both were obtained from Calbiochem (Burlington, ON, Canada).

2.4. siRNA treatment

L6 cells were transfected with 100 nM control siRNA and siRNA AMPKα1 (Sigma), using Lipofectamine 2000 (Invitrogen, 11668-019) in Optimem medium (GIBCO, 31985–070) overnight according to the manufacturer's instructions.

2.5. Mitochondrial membrane potential and ROS determination by flow cytometry

L6 myotubes were loaded with 200 nM tetramethylrhodamine (TMRM, Invitrogen, T.668) (Munoz et al., 2010) or 25 nM dihydrorhodamine-123 (Sigma, D1054) (Marambio et al., 2010), respectively, for 30 min. Fluorescence was determined by flow cytometry using a FACScan system (Becton–Dickinson).

2.6. Oxygen consumption

Cells were plated on 100 mm dishes, and when they reached confluence they were treated according to the experimental design. After treatment cells were trypsinized, and oxygen levels of the cell suspension in PBS were measured polarographically at 25 °C using a #5331 Clark electrode (Yellow Springs Instruments) (Bravo et al., 2011; Troncoso et al., 2012).

2.7. 2-NBDG uptake

L6 myotubes were serum starved for 3 h; after starvation myotubes were stimulated with insulin 100 nM for 30 min and incubated with 2-NBDG 300 μmol/L for 15 min at 37 °C as previously described (Osorio-Fuentealba et al., 2013). Cells were transferred to an Olympus Disk Scanning Unit (DSU) confocal microscope (Olympus, Hamburg, Germany). Images were quantified by ImageJ software (NIH, Bethesda, MD).

2.8. Cell-surface GLUT4*myc* quantification

Cell-surface GLUT4*myc* quantification was performed based on previously reported protocols (20). Briefly, L6 GLUT4*myc* cells were stimulated and washed twice with ice-cold PBS, fixed with 4% (v/v) paraformaldehyde for 10 min, quenched with glycine solution (100 mmol/L) for 10 min and blocked with 5% (p/v) BSA solution for 1 h. Then myotubes were incubated with anti-*myc* antibody (1:1000) overnight at 4 °C, washed 5 times with PBS and incubated with goat anti-rabbit secondary antibody (1:1000) for 1 h at 4 °C. After washing 5 times with PBS, cells were incubated with O-phenyldiamine (OPD) solution until the appearance of a yellow-

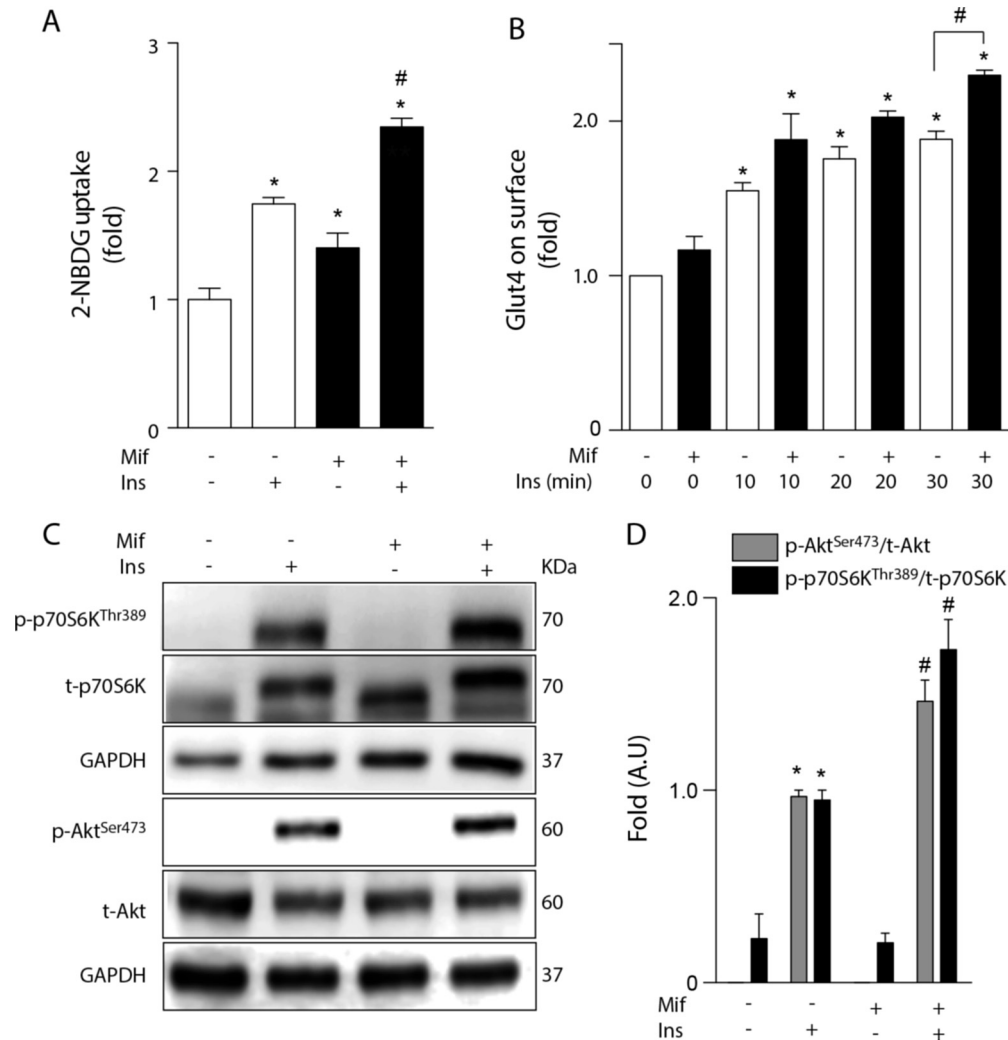


Fig. 1. Mifepristone enhances insulin response. L6 myotubes were treated with mifepristone (mif) 10 μ M for 24 h, then cells were stimulated with 100 nM insulin (ins) for 30 min (A) 2-NBDG glucose uptake in mif-treated cells (mean \pm SEM; n = 4). (B) OPD assay showing the effect of mif in GLUT4 plasma membrane translocation (mean \pm SEM; n = 4). (C) Top panel: Representative immunoblot of the effect of mif treatment in insulin-induced Akt Ser⁴⁷³ phosphorylation; Bottom panel: Densitometry analysis (mean \pm SEM; n = 4). *p < 0.05 versus control; #p < 0.05 between insulin-stimulated conditions.

red color. Absorbance was measured at 492 nm in a GloMax plate reader multi-detection system (Promega Corporation, Madison, WI, USA).

2.9. ATP measurement

Intracellular ATP content was determined using a Cell Titer-Glow Luminescent Cell Viability Assay (Promega, G7571) following manufacturer's instructions. Signals were measured in a TopCount NXT microplate luminescence counter (PerkinElmer) (Troncoso et al., 2014).

2.10. Immunofluorescence

Cell were grown on cover slips were rinsed twice with ice-cold PBS prior to fixation with 4% formaldehyde more hoechst (1:1000) in PBS for 30 min, initiated at 4 °C, and then shifted to room temperature, followed by quenching with 0.1 M glycine in PBS for 10 min. Cells were permeabilized with 0.1% Triton X-100 for 20 min at room temperature before immunolabeling with Glucocorticoid receptor (D8H2) XP Rabbit mAb (Alexa Fluor 488

Conjugate) (1:50) (Cell Signaling, Cat#12007) in PBS containing 1% BSA. The cells were rinsed six times with PBS and then water. Coverslips were mounted on slides with Dako (Carpinteria, CA) and the images were obtained on a Zeiss LSM-510 laser scanning confocal microscope.

2.11. Statistical analysis

Data are presented as mean \pm SEM of the indicated sample size (n). Student's t-test was performed to compare between two groups. Multiple groups were analyzed using one-way ANOVA followed by a protected Tukey *a posteriori* test. The GraphPad Prism 6 statistical program was used and a p value less than 0.05 was considered to represent a statistically significant difference.

3. Results

3.1. Mifepristone enhances insulin-stimulated glucose uptake

A number of diabetes-related studies have demonstrated that mifepristone restores glycemic control. Given that skeletal muscle

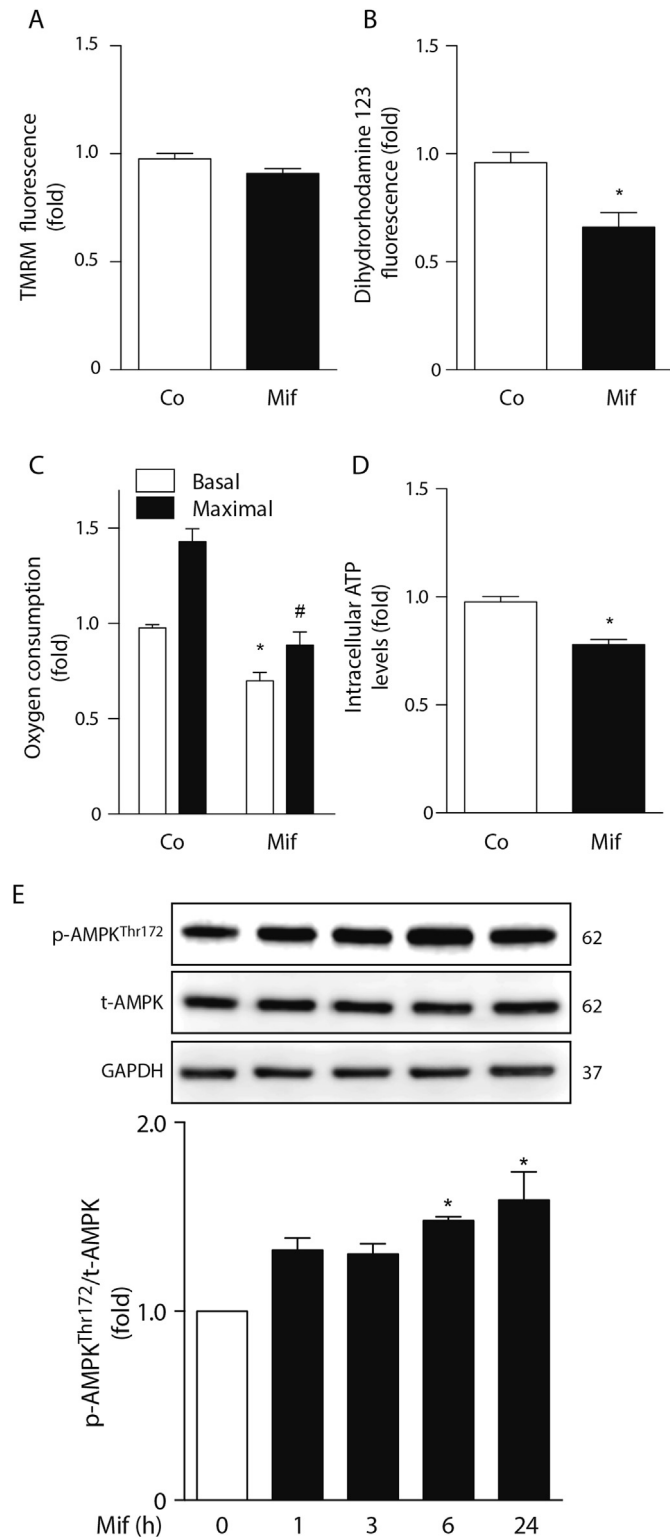


Fig. 2. Mifepristone reduces mitochondrial function. L6 myotubes were treated with mifepristone (mif) 10 μ M for 24 h. (A) Mitochondrial membrane potential was determined by TMRM staining (mean \pm SEM; n = 4). (B) Total intracellular ROS levels were measured by CM-H₂DCFDA staining (mean \pm SEM; n = 4). (C) Basal and maximal oxygen consumption rates were measured using Clark's electrode (mean \pm SEM; n = 4). (D) Intracellular ATP levels were measured with a commercial kit (mean \pm SEM; n = 4). L6 myotubes were treated with mif 10 mM for the indicated times. (E) Top panel: Representative immunoblot of the effect of mif treatment in AMPK Thr¹⁷² phosphorylation; Bottom panel: Densitometry analysis (mean \pm SEM; n = 4). *p < 0.05 versus control, #p < 0.05 versus maximal.

is responsible for glucose disposal in postprandial conditions, we proposed that mifepristone regulates insulin-dependent glucose uptake. To test this hypothesis, we used a L6 rat-derived skeletal muscle cell line stimulated for 24 h with mifepristone 10 μ M and then exposed to 100 nM insulin for 30 min. As mifepristone is a potent antagonist of glucocorticoid receptor (GR), we evaluated the GR expression and if is regulated by mifepristone. In the [supplementary Fig. 1](#), we show the expression of GR in myotubes L6, myotubes C2C12 and the HepG2 cell line ([supplementary Fig. 1A](#)). In addition, mifepristone induces the GR translocation to the nucleus in L6 cells ([supplementary Fig. 1B](#)), which is consistent with previous studies ([Jewell et al., 1995](#); [Pariante, 2004](#); [Spiga et al., 2011](#)). Moreover, mifepristone reduces the protein levels of GR ([supplementary Fig. 1C](#)). When evaluated the insulin response, our results show that mifepristone significantly increased insulin-stimulated glucose uptake evaluated through the non-metabolizable fluorescent glucose analogue 2-NBDG; we also found that mifepristone increased basal glucose uptake ([Fig. 1A](#)). The increase of glucose transport by insulin in skeletal muscle occurs by translocation to the plasma membrane of the glucose transporter GLUT4 ([Klip et al., 2014](#)). To test the participation of GLUT4 translocation on mifepristone effects, we evaluated the presence of GLUT4myc on the cell surface through the OPD assay. Mifepristone produced a significant increase in surface GLUT4myc at 30 min after insulin stimulation compared to insulin alone; no changes were observed in insulin-unstimulated conditions ([Fig. 1B](#)). The insulin signaling pathway involves the activation of serine/threonine kinase Akt ([Taniguchi et al., 2006](#)). On the basis of the aforementioned, we evaluated the effect of mifepristone on Akt Ser⁴⁷³ phosphorylation as a marker of full Akt activation ([Sarbasov et al., 2005](#)). Our results show that mifepristone significantly increases insulin-induced Akt phosphorylation at Ser⁴⁷³ ([Fig. 1C](#)), which correlates with the phosphorylation in Thr³⁸⁹ of the downstream Akt substrate p70S6K ([Fig. 1C](#)). To evaluate if components of the serum could modulate this response, we assessed the effects of mifepristone in 3 h serum-deprived L6 cells. The results showed that in serum-deprived cells, mifepristone increased Akt phosphorylation and p70S6K in response to insulin ([supplementary Fig. 2](#)). These results suggest that mifepristone improves insulin-stimulated glucose uptake in skeletal muscle cells.

3.2. Mifepristone decreases mitochondrial function and increases AMPK phosphorylation

Recent studies have demonstrated that insulin-sensitizing drugs such as metformin and the thiazolidinediones exert their metabolic beneficial effects at least in part by increasing AMPK activity ([Affourtit, 2016](#)). These effects in AMPK activity are partly due to a reduced mitochondrial function ([Affourtit, 2016](#)). To evaluate the effect of mifepristone on mitochondrial function we measured mitochondrial membrane potential, cellular ROS production, oxygen consumption and intracellular levels of ATP. We observed that mitochondrial membrane potential was not modified by a 24 h mifepristone treatment ([Fig. 2A](#)). However, mifepristone did decrease cellular ROS production ([Fig. 2B](#)), basal and maximal oxygen consumption ([Fig. 2C](#)) and ATP production ([Fig. 2D](#)) compared to control conditions. Similar results in oxygen consumption were observed with a 6 h mifepristone stimulus ([supplementary Fig. 3](#)). Next we evaluated whether the decrease in mitochondrial function was associated with the increase of AMPK Thr¹⁷² phosphorylation, as a marker of AMPK activity ([Oakhill et al., 2012](#)). Our results showed that mifepristone significantly increased AMPK phosphorylation after 6 h of stimulation ([Fig. 2E](#)), suggesting that mifepristone regulates mitochondrial function by lowering ATP levels, which could impact in AMPK activation.

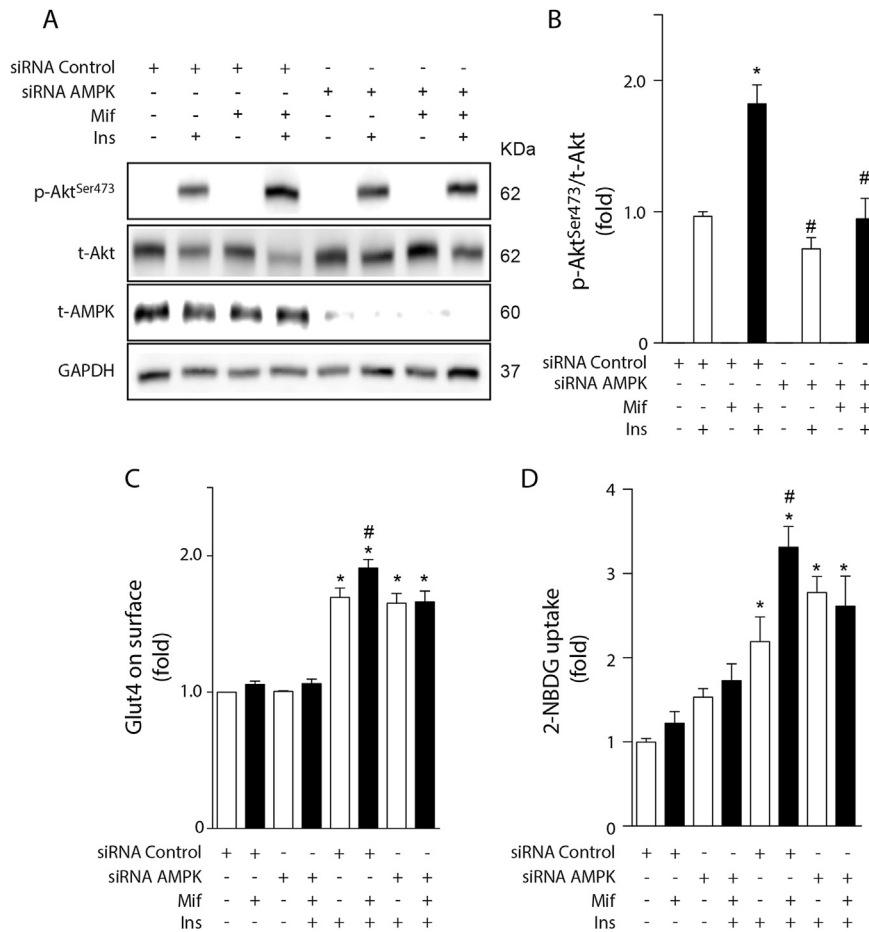


Fig. 3. Mifepristone enhances insulin-stimulated glucose uptake through AMPK. L6 myotubes were transfected with small interfering RNA of scrambled sequence (siControl) or specific siRNA against AMPK α 1 subunit sequence (siAMPK). After 48 h, cells were treated with 10 mM mifepristone (mif) for 24 h, then cells were stimulated with 100 nM insulin (ins) for 30 min. (A) Top panel: Representative immunoblot of AMPK total levels and the effect of mif treatment in insulin-induced Akt Ser⁴⁷³ phosphorylation; Bottom panel: Densitometry analysis (mean \pm SEM; n = 4). (B) OPD assay showing the effect of AMPK knockdown in GLUT4 plasma membrane translocation (mean \pm SEM; n = 4). (C) 2-NBDG glucose uptake in siAMPK-treated cells (mean \pm SEM; n = 4). *p < 0.05 versus control; #p < 0.05 between insulin-stimulated conditions.

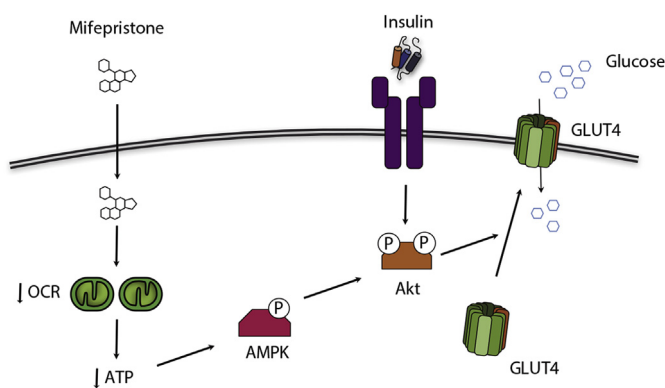


Fig. 4. Proposed model for the effect of mifepristone on insulin-stimulated glucose uptake. Mifepristone enhances the insulin cascade in the presence of insulin through a decrease of mitochondrial function that provokes a reduction in ATP production, thereby activating the sensor energy protein AMPK, which potentiates the insulin effects on Akt phosphorylation and glucose uptake.

3.3. Role of AMPK α 1 in mifepristone effects on insulin-stimulated glucose uptake

To assess the role of AMPK in the effect of mifepristone on

insulin-induced glucose uptake, the AMPK α 1 subunit was knocked down with a specific siRNA. Knockdown of the AMPK α 1 subunit blocked the effects of mifepristone on Akt phosphorylation induced by insulin (Fig. 3A–B). Moreover, AMPK α 1 siRNA prevented the effect of mifepristone on insulin-stimulated GLUT4^{myc} translocation to plasma membrane and 2-NBDG uptake (Fig. 3C–D). These results suggest that AMPK α 1 plays an important role in the enhancement of mifepristone on insulin-stimulated glucose uptake in L6 myotubes. Based on these findings, we propose that AMPK activation by mifepristone in the L6 rat skeletal muscle cell line leads to an increase of insulin response through an increase in insulin-dependent Akt phosphorylation and GLUT4 translocation to the plasma membrane.

4. Discussion

Previous studies have demonstrated that mifepristone can be useful in the glycemic control of patients with Cushing’s syndrome and type 2 diabetes. Moreover, several obesity-related animal models support the notion that mifepristone could be beneficial in the control of glycemia in different experimental settings. This is the first study that shows the direct effects of mifepristone on insulin-stimulated glucose uptake in a cellular model of skeletal muscle. In the present study, we described that mifepristone enhances insulin-stimulated glucose uptake through an AMPK-

dependent mechanism (Fig. 4).

In 1995 Kusunoki et al. showed the first evidence that in a model of insulin resistance induced by high fat diet in rats; mifepristone ameliorated insulin resistance in skeletal muscle but had no effects in heart and adipose tissue (Kusunoki et al., 1995). In addition, mifepristone reduced blood glucose levels and improved adipose insulin signaling in high fat fed-mice (Hashimoto et al., 2013). Mifepristone treatment of mature genetically obese (ob/ob) mice reduces plasma glucose levels to levels similar to those observed in lean mice (Gettys et al., 1997; Taylor et al., 2009), but without effects on beta-adrenergic signaling in adipocytes (Gettys et al., 1997). Moreover, in the obese db/db mice mifepristone also reduced glucose levels (Friedman et al., 1997; Liu et al., 2005). The same effect was reported in knockout mice for the macrophage migration inhibitory factor, which developed hyperglycemia (Nikolic et al., 2013). Additionally, in high fructose fed-mice mifepristone restored glucose levels to control levels (Priyadarshini and Anuradha, 2017). Our results show that mifepristone enhances insulin-stimulated glucose uptake through augmented translocation of GLUT4 to the plasma membrane, which could explain in part the beneficial effects of mifepristone in different animal models of insulin resistance.

Mifepristone is a potent antagonist of GR. In skeletal muscle, GCs inhibit glucose uptake and oxidation, these GCs effects antagonize those of insulin (Kuo et al., 2015). Mice treated with GCs have a reduced translocation of GLUT4 induced by insulin (Dimitriadis et al., 1997; Morgan et al., 2009; Weinstein et al., 1998). The main mechanisms reported is a decrease in IRS-1 protein levels (Giorgino et al., 1993) and the phosphorylation in serine 307 of IRS-1 (Draznin, 2006), which affect the activity of the downstream protein Akt, thus reducing the insulin response. Our result show that GR is modulate by mifepristone, however in our system we do not have an exogenous addition of GCs, this was done to evaluate the effect of mifepristone in a non-GCs stimulate system. Thus, is feasible that the effect of mifepristone on insulin signaling could be mediated by the GR, but act for an alternative via cannot be discarded.

Diverse synthetic and natural compounds have been shown to improve insulin response through a mechanism that involves the activation of AMPK (Smith and Steinberg, 2017). This protein acts as an energy sensor that responds to changes in the intracellular AMP/ATP ratio (Kahn et al., 2005). The activation of AMPK results in the stimulation of intracellular processes involved in ATP production such as glucose uptake and fatty acid oxidation and suppression of energy-consuming processes like protein and fatty acids synthesis (Kahn et al., 2005). Moreover, AMPK activation improves insulin sensitivity, and drugs that activate AMPK show therapeutic promise (Smith and Steinberg, 2017). In this regard, chronic administration of the AMPK activator 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) increased muscle insulin-stimulated glucose uptake in rats (Hawk and Richards, 1975; Jessen et al., 2003). A number of pharmacological compounds that promote insulin sensitization are able to reduce mitochondrial function, causing a decrease of intracellular ATP levels that activates AMPK (Smith and Steinberg, 2017). An example of this is given by berberine, a herb-derived class of drug widely used in China for treatment of type 2 diabetes, which induces AMPK activation through a reduction of mitochondrial function (Yin et al., 2008). Similar to berberine, thiazolidinedione and metformin do not activate AMPK directly; their effect is due to the decrease of ATP levels by the inhibition of respiratory chain complex I (Brunmair et al., 2004; Martineau, 2012). Resveratrol inhibits the mitochondrial ATP synthase by direct binding to the F1 subunit, leading to the activation of AMPK (Zang et al., 2006). In our model, we observed that mifepristone reduced mitochondrial function and

activated AMPK. Moreover, knockdown of AMPK inhibited the insulin-enhancing effects of mifepristone. Consequently, these results suggest that the mechanism of action of mifepristone in insulin response is like that of other insulin-sensitizing drugs.

Our findings support the idea of the beneficial effects of mifepristone in glycemia control. Our results suggest that mifepristone enhances insulin-stimulated glucose uptake through a mechanism that reduces mitochondrial function in skeletal muscle and promotes AMPK activation. However, further research is necessary to understand the mechanism associated with the regulation of mitochondrial function and metabolic effects of mifepristone.

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Conflict of interest

The authors have no disclosures.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.mce.2017.09.028>.

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