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Quercetin decreases inflammatory response and increases insulin action in skeletal muscle of ob/ob mice and in L6 myotubes

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ABSTRACT

Quercetin is a potent anti-inflammatory flavonoid, but its capacity to modulate insulin sensitivity in obese insulin resistant conditions is unknown. This study investigated the effect of quercetin treatment upon insulin sensitivity of ob/ob mice and its potential molecular mechanisms. Obese ob/ob mice were treated with quercetin for 10 weeks, and L6 myotubes were treated with either palmitate or tumor necrosis factor-α (TNFα) plus quercetin. Cells and muscles were processed for analysis of glucose transporter 4 (GLUT4), TNFα and inducible nitric oxide synthase (iNOS) expression, and c-Jun N-terminal kinase (JNK) and inhibitor of nuclear factor-κB (NF-κB) kinase (IκK) phosphorylation. Myotubes were assayed for glucose uptake and NF-κB translocation. Chromatin immunoprecipitation assessed NF-κB binding to GLUT4 promoter. Quercetin treatment improved whole body insulin sensitivity by increasing GLUT4 expression and decreasing JNK phosphorylation, and TNFα and iNOS expression in skeletal muscle. Quercetin suppressed palmitate-induced upregulation of TNFα and iNOS and restored normal levels of GLUT4 in myotubes. In parallel, quercetin suppressed TNFα-induced reduction of glucose uptake in myotubes. Nuclear accumulation of NF-κB in myotubes and binding of NF-κB to GLUT4 promoter in muscles of ob/ob mice were also reduced by quercetin. We demonstrated that quercetin decreased the inflammatory status in skeletal muscle of obese mice and in L6 myotubes. This effect was followed by increased muscle GLUT4, with parallel improvement of insulin sensitivity. These results point out quercetin as a putative strategy to manage inflammatory-related insulin resistance.

1. Introduction

Insulin resistance is closely related to the pathogenesis of type 2 diabetes mellitus (DeFronzo, 2004). Several experimental studies show that an inflammatory response contributes to insulin resistance in animal models of obesity (Chiang et al., 2009; Hirosumi et al., 2002; Uysal et al., 1997). This inflammatory response is elicited by cytokines such as tumor necrosis factor-α(TNFα) or by saturated fatty acids (Fernández-Real and Ricart, 2002; Groop et al., 1991; Katsuki et al., 1998). It is important to note that under insulin resistance, the adipose tissue and the skeletal muscles are important sources of TNFα (Borge et al., 2009; Borst and Conover, 2005; Furuya et al., 2010), which ultimately impairs insulin action (Plomgaard et al., 2005; Thrush et al., 2008).

Several intracellular pathways have been shown to disrupt insulin signal transmission leading to insulin resistance. Both saturated fatty acids (i.e.: palmitate) and TNFα can activate the inhibitor of nuclear factor-κB kinase (IκK), the c-Jun N-terminal kinase (JNK) (Senn, 2006; Tilg and Moschen, 2008) and the serine/threonine kinases that phosphorylate the insulin receptor substrate 1 (IRS1) and can impair insulin-stimulated serine/threonine kinase (AKT) activation and glucose transporter 4 (GLUT4) translocation (Taniguchi et al., 2006). IκK may also activate transcription factors that belong to the nuclear factor-κB (NF-κB) family, thus inhibiting GLUT4 gene transcription (Furuya et al., 2010; Ruan et al., 2002).

Quercetin is an abundant flavonoid (Crozier et al., 1997) with a well-recognized potent and non-selective anti-inflammatory action that relies on a wide range of mechanisms of action.

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For instance, quercetin was described to inhibit NF-κB activity, inducible nitric oxide synthase (iNOS) expression (Dias et al., 2005) and lipoxygenase activity (Hu et al., 2006). It has been demonstrated that quercetin administration diminishes hyperglycemia in rats rendered diabetic by streptozotocin injection (Vessal et al. 2003). However, quercetin action is known to protect pancreatic islets that certainly favors glycemic control in streptozotocin-induced diabetes (Coskun et al., 2005; Kim et al., 2007).

In contrast to what was found in streptozotocin-induced diabetes, the effects of quercetin in obese rodents have originated controversial data. Addition of quercetin to high-fat diet did not prevent obesity-induced insulin resistance in mice (Stewart et al., 2009). However, intragastric quercetin improved whole body insulin sensitivity and reduced iNOS expression in the liver of obese rats (Rivera et al., 2008).

Considering the relevance of definitely determining whether the natural compound quercetin has any effect upon insulin resistance during the development of obesity, the present study investigated the effects of quercetin on inflammatory status and insulin sensitivity of ob/ob mice. The results showed that quercetin reduced whole-body insulin resistance and decreased inflammatory pathways in skeletal muscle. In addition, the relationship between the anti-inflammatory property of quercetin, and its ability to improve insulin action in skeletal muscle was further evidenced by in vitro experiments with L6 myotubes challenged with palmitate and TNFα.

2. Materials and methods

2.1. Animals and treatment

Recessive homozygote ob/ob mice aging approximately 3 months were obtained from the animal care facility of the Federal University of Sao Paulo (CEDEME-UNIFESP) and the experimental protocol was approved by the Ethical Committee for Animal Research of the Institute of Biomedical Sciences of the University of Sao Paulo. Animals (four per cage) had free access to standard chow (Nuvilab CR-1; Nuvital, Curitiba, Brazil) and water, and were maintained at 23 °C on a 12 h light/dark cycle. Mice were divided into two groups that received either quercetin or vehicle. Quercetin was suspended in distilled water (15 mg/ml), and injected intraperitoneally (i.p.) every other day for 10 weeks at the dosage of 30 mg/kg. Vehicle- and quercetin-treated mice were killed at the end of the tenth week of treatment, and respectively designated as CTL and Quer throughout the manuscript.

2.2. Insulin tolerance test (ITT)

Insulin (2 U/kg) was administered i.p., and tail blood samples were collected before and at 10, 20, 30, 40 and 60 min after insulin injection for blood glucose determination. The rate constant for glucose disappearance during insulin tolerance test (K_{ITT}) was calculated using the formula $0.693/t_{1/2}$.

2.3. L6 cell culture and treatments

L6 myoblast were maintained in DMEM containing 10% Fetal Bovine Serum (FBS), 25 mM glucose, penicillin (100 IU) and streptomycin (100 μg/ml) in a humidified atmosphere with 5% CO_2 at 37 °C. L6 myoblasts (80% confluent) were allowed to differentiate into myotubes in DMEM containing 2% FBS for 4–6 days. Quercetin (diluted in ethanol) was added 48 h prior to the experiments at a final concentration ranging from 5 to 200 μM. Palmitate treatment (750 μM) of myotubes was carried out during the last 20 h of quercetin treatment in DMEM containing 1% Bovine Serum Albumin (BSA). TNFα treatment (20 ng/ml) was carried out during the last 6 h of quercetin treatment. Acute insulin challenge of myotubes (100 nM, 10 min.) was performed in Krebs–Henseleit after a 45-min pre-incubation at 37 °C under a 95% O_2:5%CO_2 atmosphere. After stimulus, myotubes were rapidly harvested in ice-cold PBS and pulled down to be processed for further experiments.

2.4. Protein extraction and western blotting

Gastrocnemius muscles and myotubes were processed for Western blotting as previously described (Anhê et al., 2007). Membranes were probed with antibodies against phospho-IκK (Cell Signaling Technology, Danvers, MA, USA), phospho-JNK, phospho-AKT1/2/3 (Ser 473) (from Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β-actin (Millipore, Billerica, MA, USA). Chemiluminescent detection was performed after incubation with a secondary antibody conjugated to HRP (GE Healthcare, Buckinghamshire, England). Band intensities were visualized in X-ray sensitive films and quantified by using Scion Image software (Scioncorp, USA).

2.5. RNA isolation and RT-PCR

Total RNA was extracted from 100 mg of mice gastrocnemius and L6 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized using InProm Reverse Transcriptase (Promega, Madison, WI, USA). cDNA was used for end-point conventional (GoTaq DNA Polymerase, Promega, Madison, WI, USA) or real-time (SYBR Green master mix, Applied Biosystems, Foster City, CA, USA) PCR. For the L6 samples end-point, PCR was performed as already described (Bordin et al., 2004), and the results were normalized by *gapdh* expression. For the muscle samples, real-time PCR was performed, and the relative expression was analyzed using the 2(-Delta Delta C(T)) method (Livak & Schmittgen, 2001). The primer sequences used, as well as annealing temperatures, are described in Supplementary tables 1 and 2 available as Supporting material.

2.6. Total membrane fractioning and GLUT4 detection

Cells and muscles were mixed with homogenization buffer and processed as previously described (Anhê et al., 2007). Homogenates were centrifuged at 700 × *g* at 4 °C for 10 min. The pellets containing protein from membrane fractions were obtained from the supernatants (Anhê et al., 2007). The intensity of the bands representing GLUT4 was quantified by densitometry and normalized by the densitometric scanning of coomassie brilliant blue (CBS) stained gel using the whole lane for each sample. Afterwards, taking into account the total protein yield from each muscle the results were expressed as total GLUT4 (AU) per whole tissue.

2.7. Nuclear translocation of NF-κB proteins

L6 myotubes were suspended in cell lysis buffer (10 mM Tris pH 8.0, 10 mM NaCl, 0.2% Nonidet-P40) containing phosphatase and protease inhibitors (sodium orthovanadate, phenylmethylsulphonyl floride and aprotinine). After 10 min incubation on ice, the samples were centrifuged at 2,500 x*g* for 15 min at 4 °C. The pellets of nuclei were suspended in nuclear lysis buffer (20 mM tris pH 8.0, 10 mM EDTA, 1% SDS and phosphatase and protease inhibitors). Afterwards, the samples were homogenized by sonication and processed as for Western blot. The nitrocellulose membranes with nuclear proteins were probed with antibodies

against c-Rel, p50 and p52 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Chemiluminescent detection was performed as described in Western blot section. Membranes were stained with ponceau in order to check for equal protein loading in all experiments.

2.8. Chromatin Immunoprecipitation (ChIP) assay

Gastrocnemius muscles from ob/ob mice were processed using buffers and reagents from EZ ChIP Chromatin Immunoprecipitation Kit (Upstate, Lake Placid, NY, USA) according to the manufacturer instructions. Briefly, muscles were removed and minced in PBS containing 1% formaldehyde. Next, tissue fragments were incubated for 10 min at room temperature and transferred to Lysis Buffer. DNA was sheared to fragments of approximately 200–1000 bp by applying eight bursts of sonication (20% of power, 10 s each). Samples were diluted in Dilution Buffer and pre-cleared for 1 h at 4 °C with protein *G*-agarose (50% slurry) (GE Healthcare, Buckinghamshire, England) saturated with salmon sperm DNA. An aliquot of 10 μl was collected as "input". The remaining supernatants were subjected to immunoprecipitation with protein G-agarose saturated with salmon sperm DNA and 5 μg of anti-p50 NF-κB antibody (Santa Cruz Biotechnology, sc-26418). In parallel, one sample was incubated with protein G-agarose only in order to generate the negative control (no-AB). Agarose pellets were then washed with buffers provided in the kit and treated with elution buffer. Supernatants were subjected to crosslinking reversal and RNase A treatment. DNA was purified using phenol-chloroform and resuspended in 20 μl of ddH_2O. DNA samples were amplified for detection of *Slc2a4* promoter. Primers spanning the GLUT4 5′ region from −151 to +73 were 5′–TTCCGTGGGTTGTGGCAGTG-3′ and 5′–ACCAGTG-TTCCAGTCACTCGCTG-3′. The products were amplified by real time PCR, as described in the previous section, using 3.0 mM of $MgCl_2$, 2 μl of template DNA and 55 °C of annealing temperature. The input values from each sample were used as inner control and the 2(-Delta Delta C(T)) formula was used to calculate relative binding of NF-κB p50 to GLUT4 promoter. After reaction, the products were run on a 2% agarose gel and a digital image was acquired.

2.9. 2-Deoxy-[2,6−^{3}H]$_D$-glucose (DG) uptake

Myotubes seeded in six-well plates were treated as previously described and pre-incubated for 6 h in DMEM without serum and glucose. DMEM was removed and cells were washed and pre-incubated with Krebs-Henseleit at 37 °C for 10 min with or without insulin (100 nM). The buffer was replaced by a fresh one containing 5.6 mM glucose and 0.2 mCi/ml 2-DG, and cells were incubated for 20 min at 37 °C. Next, cells were washed in iced-cold PBS and the radioactivity associated with the cells was determined after lysis with 1 M KOH at 70 °C, followed by liquid scintillation of aliquots (100 μl). The uptake measurement was performed in triplicate. Protein concentration of each sample was determined by the Bradford method. Results were normalized by mg of total protein.

2.10. Data analysis

All values are mean ± S.E.M.. Means from both cells and mice experiments were statistically analyzed by unpaired Student's *t*-test or one-way ANOVA, with Newman–Keuls post-test

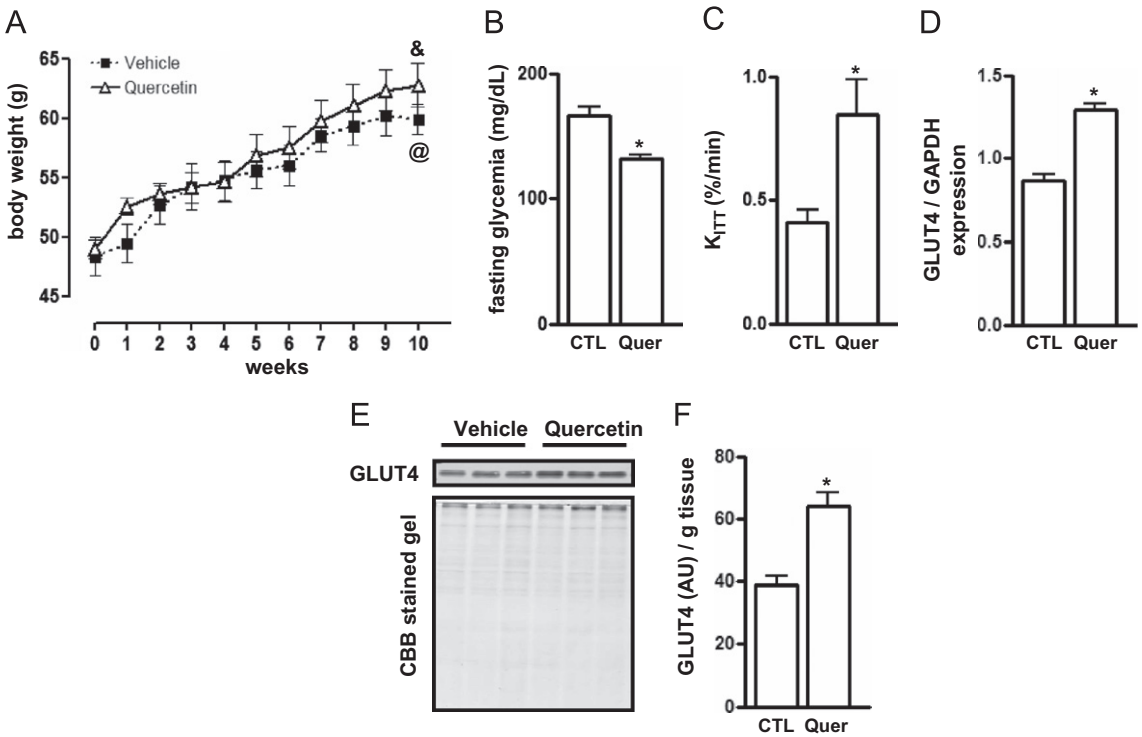

Fig. 1. Intraperitoneal quercetin treatment increases insulin sensitivity in ob/ob mice. Obese ob/ob mice were treated either with quercetin (Quer) or vehicle (CTL) for 10 weeks. Mice were weighed weekly (A) and fasting glycemia was measured at the end of the tenth week (B). The glucose decay phase was used for the calculation of the K_{ITT} from data acquired after intraperitoneal insulin tolerance test (C). Gastrocnemius muscles were used for GLUT4 mRNA quantification by real time PCR (D). Gastrocnemius muscles were processed for total membrane fractioning and GLUT4 detection by Western blot (E–upper panel). The intensity of the blots was normalized to the coomassie blue stained (CBS) gel (E – lower panel) and expressed as total GLUT4 (AU) per whole tissue (F). Data are presented as mean ± S.E.M.. * $P<0.05$ vs. CTL; & $P<0.05$ vs. mice before the beginning of treatment with quercetin; @ $P<0.05$ vs. mice before the beginning of treatment with vehicle ($n=6$).

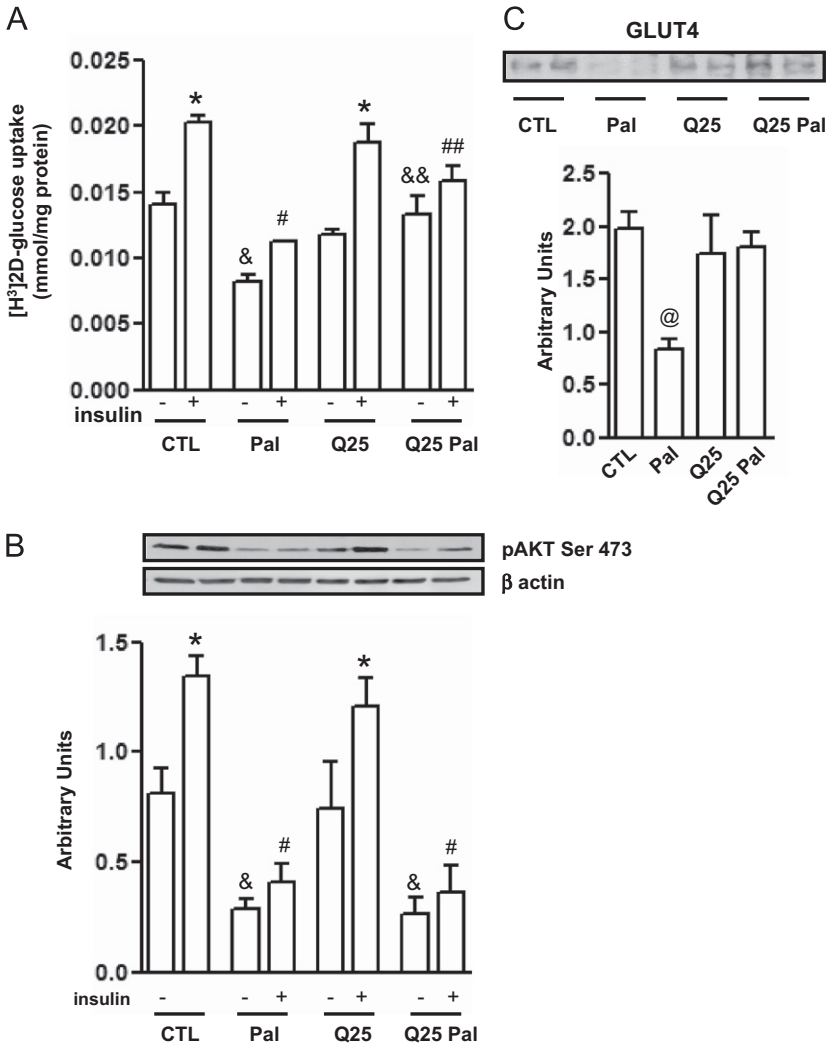

Fig. 2. Quercetin prevents downregulation of GLUT4 expression and insulin-induced glucose uptake elicited by palmitate but does not modulate Akt serine phosphorylation. L6 myotubes were treated with 25 μM quercetin and/or 750 μM palmitate (Pal) as described in the section 2.3. After treatment, myotubes were incubated in Krebs buffer and acutely (10 min.) stimulated with insulin (100 nM) or vehicle (NaCl 0.9%). Non-stimulated and insulin-stimulated L6 myotubes were processed for 2-DG uptake assay (A) and Western blot detection of serine 473 phosphorylated Akt1/2/3, which was normalized to β actin (B). Myotubes treated with quercetin (Q25) and/or palmitate (Pal) were also used for total membrane extraction and GLUT4 detection by Western blot (C). Data are presented as mean ± S.E.M.. $^{*}P<0.05$ vs. non insulin-stimulated myotubes within the same condition; $^{\#}P<0.05$ vs. insulin-stimulated CTL; & $P<0.05$ vs. non insulin-stimulated CTL; $^{\#\#}P<0.05$ vs. insulin-stimulated Pal; && $P<0.05$ vs. non insulin-stimulated Pal; @ $P<0.05$ vs. CTL ($n=5$ for 2-DG uptake and phospho Akt and $n=6$ for GLUT4).

(INStat - Graph Pad Software, Inc., San Diego, USA). The level of significance was set at $P<0.05$.

3. Results

3.1. Quercetin treatment decreases fasting glycemia and insulin resistance and increases GLUT4 expression in skeletal muscle in ob/ob mice

Our first experimental approach showed that quercetin treatment did not affect body weight gain (Fig. 1A). However, quercetin reduced fasting glycemia to values close to 88% of those from vehicle-treated ob/ob mice after 10-week treatment (Fig. 1B), and that must have been the consequence of insulin sensitivity increasing in ob/ob treated-mice, in such a way that K_{ITT} values of Quer mice was 206% of those from CTL mice (Fig. 1C).

Fig. 1D shows that GLUT4 mRNA was upregulated in skeletal muscle of ob/ob mice treated with quercetin (to 149% of CTL mice values); and that was accompanied by a parallel increase of 154% in GLUT4 protein content (Figs. 1E and F).

3.2. Downregulation of GLUT4 expression and insulin-induced glucose uptake induced by palmitate are prevented by quercetin without changes in AKT serine phosphorylation

Palmitate decreased 2-DG uptake in insulin stimulated (to 54% of insulin-stimulated CTL values) and non stimulated L6 myotubes (to 58% of non-stimulated CTL values). Quercetin (25 μM) prevented this effect of palmitate (Fig. 2A). Non-stimulated and insulin-stimulated glucose uptake in cells treated with quercetin plus palmitate were, respectively, 162% and 145% of that from cells treated with palmitate only. However, cells treated with quercetin plus palmitate remained insulin resistant. Insulin-stimulated AKT serine phosphorylation was also reduced by palmitate treatment (to 30% of insulin stimulated-stimulated CTL values) which was not recovered by quercetin (Fig. 2B). GLUT4 protein content was reduced in cells treated with

palmitate (to 54.8% of CTL values) but the addition of quercetin prevented this downregulation (Fig. 2C). In these experiments, quercetin alone did not affect any of these parameters (Figs. 2A, B and C).

3.3. Downregulation of insulin-induced glucose uptake and AKT serine phosphorylation by TNFα are prevented by quercetin

Insulin-stimulated 2-DG uptake in L6 myotubes was significantly reduced by the addition of TNFα (to 33.4% of insulin-stimulated CTL values). Quercetin, when added prior to TNFα, prevented the decrease in insulin-stimulated 2-DG uptake. In fact, insulin-stimulated 2-DG uptake in cells treated with quercetin combined or not with palmitate was approximately 200% higher than that of CTL cells. Thus, in these experiments, quercetin itself was able to increase insulin-stimulated 2-DG uptake irrespective of the presence of TNFα (Fig. 3A). Insulin-stimulated AKT serine phosphorylation was reduced by TNFα (to 37.4% of insulin-stimulated CTL values), and quercetin avoided this reduction (Fig. 3B).

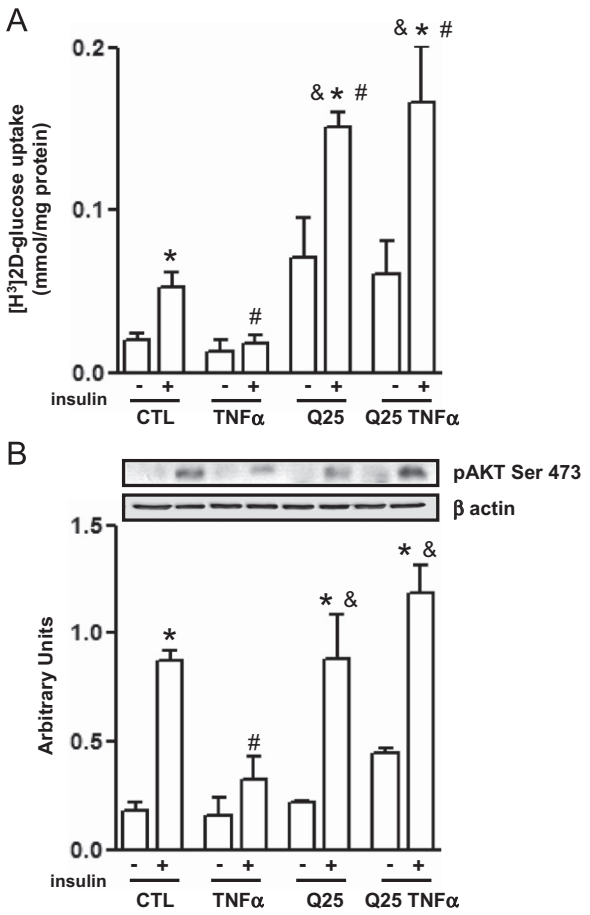

Fig. 3. Quercetin prevents downregulation of insulin-induced glucose uptake and Akt serine phosphorylation elicited by TNFα. L6 myotubes were treated with 25 μM quercetin and/or TNFα (20 ng/ml) as described in the section 2.3. After treatment, myotubes were incubated in Krebs buffer and acutely (10 min.) stimulated with insulin (100 nM) or vehicle (NaCl 0.9%). Non-stimulated and insulin-stimulated L6 myotubes were processed for 2-DG uptake assay (A) and Western blot detection of serine 473 phosphorylated Akt1/2/3, which was normalized to β actin (B). Data are presented as mean ± S.E.M.. $^{*}P<0.05$ vs. non insulin-stimulated myotubes within the same condition; $^{\#}$ $P<0.05$ vs. insulin-stimulated CTL; & $P<0.05$ vs. insulin-stimulated TNFα ($n=5$ for 2-DG uptake and phospho Akt).

3.4. Quercetin treatment modulates the expression of TNFα and iNOS in muscle

Quercetin reduced TNFα mRNA levels in muscle of ob/ob mice (to 48% of CTL mice values) (Fig. 4A). Similarly, quercetin reduced iNOS expression in muscle of ob/ob mice (to 57% of CTL mice values) (Fig. 4B). In L6 myotubes, palmitate increased TNFα expression (to 192.3% of CTL values), and quercetin (5 and 25 μM) suppressed this upregulation. Higher doses of quercetin, such as 100 and 200 μM, failed to avoid this effect of palmitate (Fig. 4C). Similarly, palmitate stimulated iNOS expression in L6 myotubes (to 189% of CTL values), and quercetin (25 μM) abolished this effect. Lower (5 μM) and higher (100 and 200 μM) doses of quercetin also failed to modulate the effect of palmitate on iNOS expression (Fig. 4D).

3.5. Quercetin treatment decreases JNK but not IκK phosphorylation in muscle cells

Treatment with quercetin decreased JNK phosphorylation in skeletal muscle of ob/ob mice (to 53% of CTL mice values) (Fig. 5B) but did not change IκK phosphorylation (Fig. 5C). JNK phosphorylation was also stimulated in L6 myotubes treated with palmitate for 6 h (to 225% of CTL values), and quercetin suppressed this effect (Fig. 5D). IκK phosphorylation was not modulated in L6 myotubes treated with palmitate and/or quercetin (Fig. 5F). L6 myotubes treated with TNFα also displayed an increase in JNK phosphorylation (to 168% of CTL values) which was abolished by quercetin (Fig. 5E). TNFα increased IκK phosphorylation in L6 myotubes (to 202% of CTL values) which was not modulated by quercetin (Fig. 5G).

3.6. Quercetin modulates nuclear accumulation of NF-κB and NF-κB p50 binding to GLUT4 promoter in skeletal muscle cells

Palmitate-induced nuclear p50 accumulation in L6 myotubes (to 152% of CTL values) was abolished by quercetin (Fig. 6A). Palmitate has also stimulated c-Rel accumulation in the nucleus (to 585% of CTL values), which was suppressed by quercetin (Fig. 6B). Palmitate-induced nuclear p52 accumulation (to 277% of untreated myotubes values) was not altered by quercetin (Fig. 6C). We have also found that ob/ob mice treated with quercetin had reduced NF-κB p50 binding to GLUT4 promoter in skeletal muscle (to 12% of CTL mice values) (Fig. 6D).

4. Discussion

It has been a consensus that quercetin decreases glycemia in streptozotocin-diabetic rats (Coskun et al., 2005; Vessal et al. 2003); however, since this flavonoid has a protective effect in pancreatic β-cell (Coskun et al., 2005; Kim et al., 2007), that may be a result of decreased severity of diabetic condition. The present study shows that intraperitoneal treatment of ob/ob mice with quercetin reduces whole-body insulin resistance by acting in skeletal muscle. A previous study showed that orally given quercetin improved insulin sensitivity in Zucker rats (Rivera et al., 2008), although two groups have demonstrated that quercetin, when mixed with a high-fat diet or when given by gavage did not ameliorate insulin resistance (Romero et al., 2010; Stewart et al., 2009). Failure to detect a consistent effect of quercetin when orally administrated might probably result from its low intestinal absorption (Nait Chabane et al., 2009) and to its high metabolization by the enterocytes (Rechner et al., 2002). This way, only 5.3% of the quercetin orally administrated reaches blood stream in its original form (Rechner et al., 2002). Thus, our

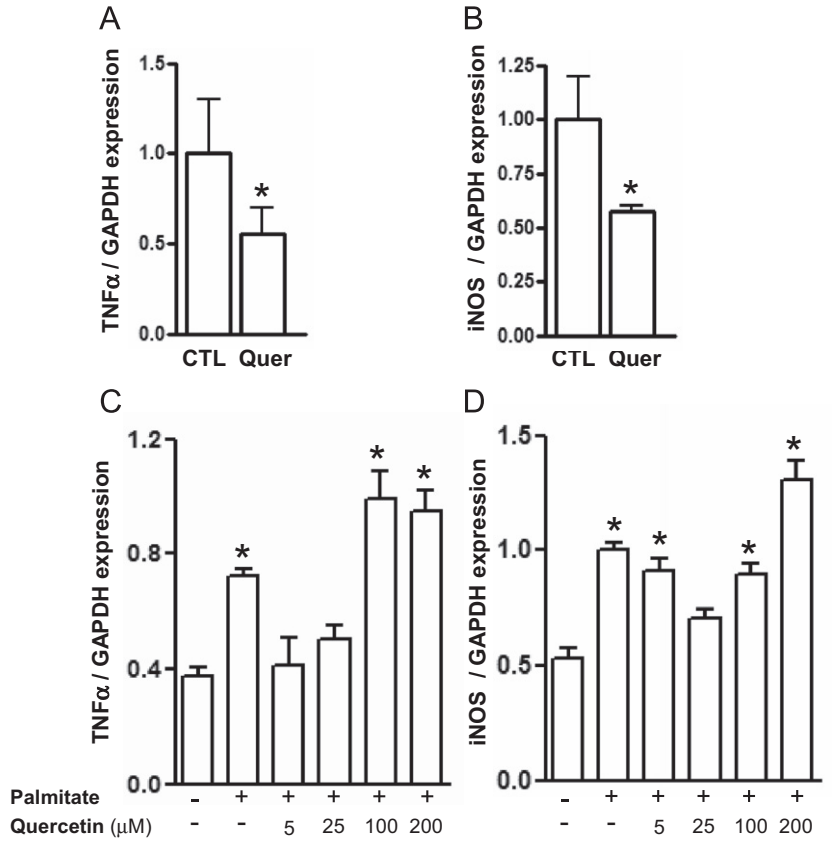

Fig. 4. Quercetin modulates the expression of TNFα and iNOS in skeletal muscle of ob/ob mice and in L6 myotubes treated with palmitate. Obese ob/ob mice were treated either with quercetin (Quer) or vehicle (CTL). At the end of week 10 of treatment, mice were sacrificed and gastrocnemius muscles were used for quantification of TNFα (A) and iNOS (B) mRNAs by Real time PCR. L6 myotubes were treated with palmitate (750 μM) combined or not with quercetin (5, 25 100 and 200 μM). After treatment, cells were used for TNFα (C) and iNOS (D) mRNAs quantification by conventional PCR. Data are presented as mean ± S.E.M.. * $P < 0.05$ vs. CTL mice or myotubes ($n=5$ for mice data and $n=6$ for myotubes data).

present data recover the potential of quercetin to treat insulin resistance, mainly by routes that bypass the gut barriers. In order to clarify the mechanism by which quercetin improves insulin sensitivity, we next moved on to experiments with skeletal muscles of obese mice and with L6 myotubes. Importantly, the dose of quercetin used in our in vivo experiments was very similar to those used to describe the effect of quercetin in streptozotocin-diabetic rats (Dias et al., 2005; Coskun et al., 2005; Vessal et al. 2003).

Skeletal muscle was chosen as a putative target for quercetin because glucose uptake by this tissue accounts for the majority of glucose disposal in the post-prandial period, as well as after a challenge with exogenous insulin. As the glucose transporter GLUT4 is the limiting step for glucose uptake (Wallberg-Henriksson and Zierath, 2001), we initially investigated the GLUT4 expression in skeletal muscle of obese mice. We have found that quercetin increased GLUT4 content in skeletal muscle of ob/ob mice after 10 weeks of treatment, favoring the hypothesis that quercetin action in skeletal muscle might account for the increase in whole body insulin sensitivity.

To investigate the mechanism of action of quercetin in skeletal muscle, we next used L6 myotubes challenged with palmitate or TNFα, very well known to be inducers of insulin resistance in vitro (Steinberg et al., 2006). At this point, it is important to stress the differences between the effects of quercetin in L6 myotubes treated with palmitate or TNFα. Quercetin was able to increase the levels of 2-DG uptake in myotubes treated with palmitate; however, cells treated with quercetin plus palmitate did not recover insulin sensitivity. In contrast, when TNFα was used to induce insulin resistance, quercetin restored insulin sensitivity. These differences are in accordance with our findings of AKT phosphorylation. In these experiments, we found that the impairment of insulin-stimulated AKT phosphorylation by TNFα was completely avoided by quercetin, which was not seen in palmitate-treated cells. Thus, restoration of insulin-induced AKT phosphorylation in TNFα-treated cells might explain the ability of quercetin to recover insulin-sensitivity, as assessed by 2-DG uptake. The increase in 2-DG uptake levels (whether basal or insulin-stimulated) induced by quercetin in palmitate-treated cells is, therefore, likely to result from a restoration of GLUT4 levels. Although a previous study has shown that quercetin increases insulin-induced glucose uptake by adipocytes (Fang et al., 2008), the present study is the first to experimentally evidence that quercetin facilitates insulin action in skeletal muscle and to relate this action to a modulation of GLUT4 expression.

Another important observation refers to the action of quercetin over 2-DG uptake in the absence of palmitate and TNFα. Quercetin itself did not modulate 2-DG uptake in the experiments using palmitate to induce insulin resistance, however, in the experiments that we used TNFα, the sole presence of quercetin increased 2-DG uptake. This difference might be due to the medium used in each experiment. Palmitate treatment requires a serum-free medium with 1% BSA, which was used in all experimental groups. In the case of experiments using TNFα, all cells were treated with medium containing 10% FBS. Thus, it is possible that quercetin synergizes with some serum component to increase 2-DG uptake. This can be concluded because quercetin by itself, when used in serum-free medium, did not increased 2-DG uptake.

The reason why quercetin overcomes TNFα- but not palmitate-induced insulin resistance in muscle might be due to the different

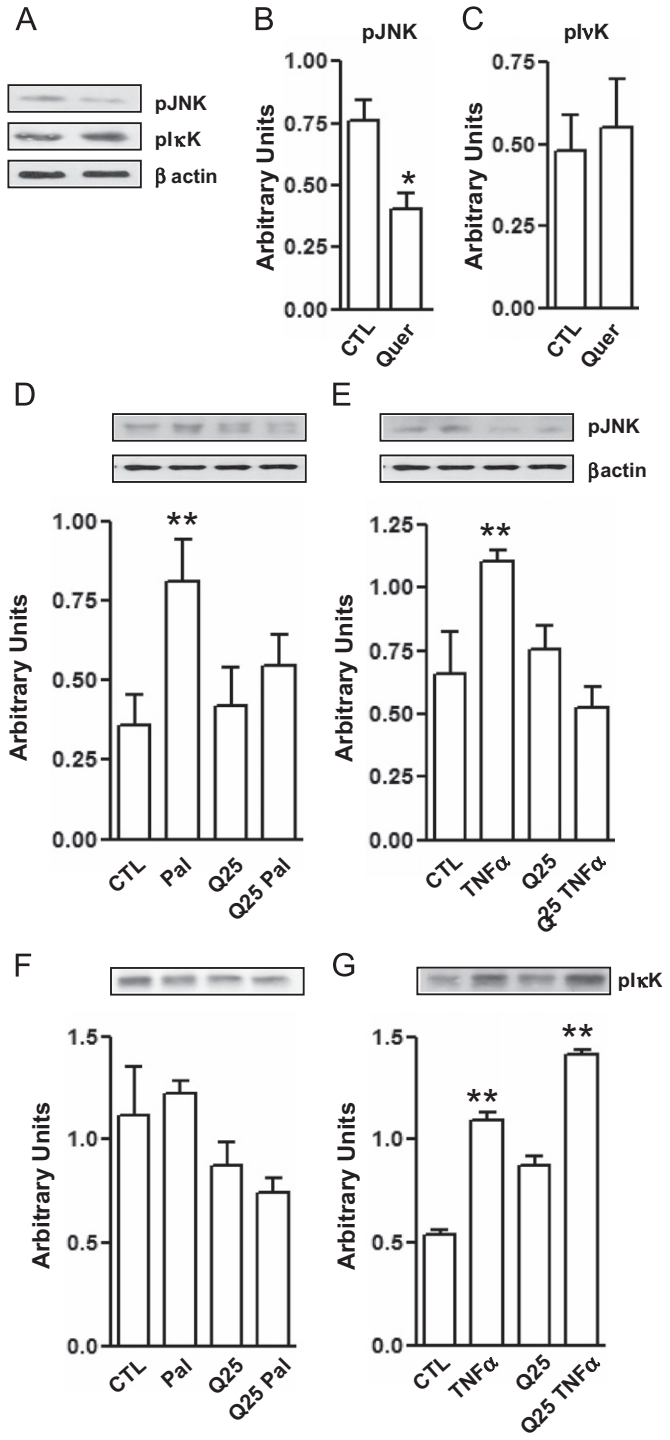

Fig. 5. Quercetin modulates the phosphorylation of JNK but not IκK in skeletal muscle of ob/ob mice and in L6 myotubes treated with palmitate and TNFα. Obese ob/ob mice were treated either with quercetin (Quer) or vehicle (CTL). At the end of week 10 of treatment, mice were sacrificed and gastrocnemius muscles were used to Western blot detection of pJNK, pIκK and β actin (A). Values of pJNK (B) and pIκK (C) were normalized to those of β actin. L6 myotubes were treated with quercetin 25 μM (Q25) and/or palmitate (Pal) as described in Section 2.3. After treatment, cells were processed for Western blot detection of pJNK (D) and pIκK (F) which were normalized to β actin. L6 myotubes were also treated with quercetin (Q25) and or TNFα as described in the section 2.3. After treatment, cells were processed for Western blot detection of pJNK (E) and pIκK (G) which were normalized to β actin. Data are presented as mean ± S.E.M.. * $P<0.05$ vs. CTL mice; ** $P<0.05$ vs. CTL myotubes ($n=4$).

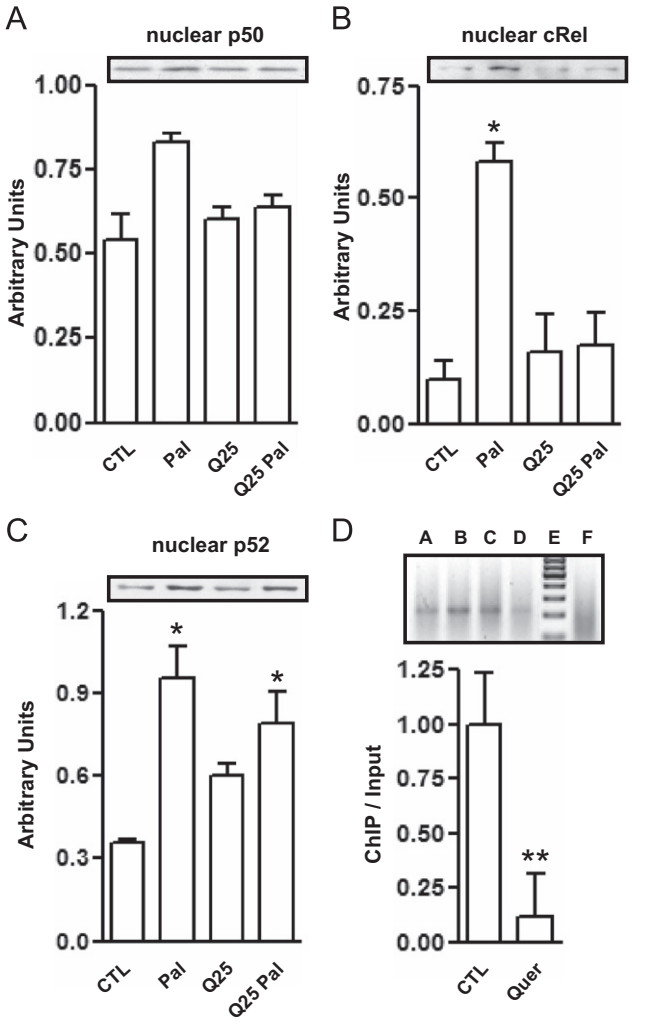

Fig. 6. Quercetin modulates the nuclear accumulation of NF-κB proteins in L6 myotubes treated with palmitate and NF-κB binding to GLUT4 promoter in skeletal muscle of ob/ob mice L6 myotubes were treated with 25 μM quercetin (Q25) and/or palmitate (Pal) as described in Section 2.3. After treatment, protein from nuclear fraction was extracted and subjected to Western blot detection of p50 (A), cRel (B) and p52 (C). Obese ob/ob mice were treated either with quercetin (Quer) or vehicle (CTL) and their gastrocnemius muscles were removed and processed for ChIP assay using anti- NF-κB p50 antibody. Samples were subjected to real time PCR analysis of GLUT4 promoter and each sample was normalized to the respective input. The products were run on an agarose gel. The line are as follows: A-representative input from CTL mice; B-representative input from Quer mice; C-representative ChIP from CTL mice; D-representative ChIP from Quer mice; E-100 bp ladder; F-no-AB (D). Data are presented as mean ± S.E.M. * $P<0.05$ vs. CTL myotubes; ** $P<0.01$ vs. CTL mice ($n=6$ for nuclear extracts and $n=4$ for ChIP assay).

mechanisms by which these agents induce insulin resistance. TNFα is widely known to stimulate insulin resistance by activating JNK (Aguirre et al., 2000). Concordantly, we found that quercetin efficiently abrogated TNFα- and palmitate-induced JNK phosphorylation. However, palmitate-induced insulin resistance is likely to result from additional metabolic mechanisms such as ceramides generation, which are known to reduce AKT activation (Sabin et al., 2007). These additional effects of palmitate leading to insulin resistance are probably not being modulated by quercetin in skeletal muscle cells.

Insulin resistance in obese models is believed to result from the increased circulating or local production of inflammatory cytokines, as well as from the increased circulating or local accumulation of saturated free fatty acids (FFA) (Plomgaard et al., 2005; Thrush et al., 2008). In addition, FFA might exert a cooperating effect by increasing the expression of inflammatory cytokines such as TNFα, and iNOS in skeletal muscle (Jové et al., 2006). Nitric oxide (NO) resultant from iNOS activity has been

described to induce S-nitrosation of IRS1, thus blunting tyrosine phosphorylation (Carvalho-Filho et al., 2005). Here, we further demonstrate that quercetin reduces TNFα and iNOS expression in skeletal muscle of ob/ob mice after 10 weeks of treatment. This was also found in palmitate-treated myotubes. These results are in agreement with a previous report demonstrating that quercetin reduces circulating TNFα and iNOS expression in adipose tissue of Zucker rats (Rivera et al., 2008).

It is important to emphasize that the ability of quercetin in decrease palmitate-stimulated TNFα and iNOS expression was restricted to doses lower than 100 μM. These findings are in accordance with studies describing that an anti-inflammatory action of quercetin comprises doses ranging from 10 to 40 μM. These moderate concentrations of quercetin are able to inhibit TNFα and iNOS expression and NF-κB activity in several cell types. In spite of this, several studies demonstrate that higher doses (above 100 μM) of this flavonoid stimulate apoptosis in several cancer cell types. This effect of quercetin is mediated by the generation of Reactive Oxygen Species (ROS) and inactivation of AKT. Thus, in accordance with published data, our results show that higher doses of quercetin, combined with palmitate, are predominantly oxidative/inflammatory (increasing TNFα and iNOS expression in L6 myotubes) (Granado-Serrano et al., 2006; Senthilkumar et al., 2010).

Apart from inhibiting insulin signaling, the inflammatory response elicited by palmitate and TNFα might also induce insulin resistance by reducing GLUT4 expression in muscle and adipose cells through NF-κB activation (Furuya et al., 2010; Jové et al., 2006; Silva et al., 2005; Ruan et al., 2002). Accordingly, a recent report has demonstrated that whole body NF-κB p50 deletion increases insulin sensitivity by upregulating insulin action in both liver and skeletal muscle (Gao et al., 2009). According to these studies, we now show that quercetin is able to recover GLUT4 expression abrogated by palmitate, and to suppress palmitate-induced NF-κB accumulation in the nucleus. Besides, we demonstrate that quercetin reduces the in vivo binding of NF-κB p50 to GLUT4 promoter in skeletal muscle of ob/ob mice. Together, these data suggest that the anti-inflammatory action of quercetin, hallmarked by a reduction in NF-κB activity, is related to the improvement of GLUT4 expression in muscle cells.

In order to focus on the mechanism by which quercetin modulates NF-κB activity, we assessed IκK phosphorylation. Under non-stimulated conditions, NF-κB is inactive in the cytoplasm bound to its inhibitory protein inhibitor of NF-κB (IκB). Following stimulus, a signaling cascade is triggered resulting in the activation of IκK. This kinase leads to phosphorylation and degradation of the IκB, releasing NF-κB to the nucleus (Gilmore, 1999). The mechanism by which quercetin downregulates NF-κB nuclear translocation and binding activity in muscle cells, however, does not seem to involve a reduction in IκK phosphorylation. Importantly, inhibitory effect of quercetin over NF-κB has already been described to occur through decreased IκB degradation (Ying et al., 2009).

The present results demonstrate that intraperitoneal quercetin treatment improves insulin sensitivity in ob/ob obese mice. Considering the data presented herein, it is reasonable to hypothesize that the downregulation of both JNK and NF-κB by quercetin might act synergistically in skeletal muscle to reduce palmitate and TNFα action increasing whole-body insulin sensitivity. Favoring this hypothesis, both JNK and NF-κB inhibition have been described to mediate the anti-inflammatory properties of other flavonoids such as resverastrol (Kundu and Surh, 2004). Quercetin suppression of NF-κB activation and, by extension, binding to the GLUT4 promoter is probably involved in the upregulation of GLUT4 expression. These results point out the potential role of quercetin for the development of new therapeutic approaches to manage insulin resistance.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejphar.2012.06.007.

References

Aguirre, V., Uchida, T., Yenush, L., Davis, R., White, M.F., 2000. The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). J. Biol. Chem. 275, 9047–9054.

Anhê, G.F., Hirabara, S.M., Turrer, T.C., Caperuto, L.C., Anhê, F.F., Ribeiro, L.M., Marçal, A.C., Carvalho, C.R., Curi, R., Machado, U.F., Bordin, S., 2007. Postpartum glycemic homeostasis in early lactating rats is accompanied by transient and specific increase of soleus insulin response through IRS2/AKT pathway. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292, R2225–2233.

Bordin, S., Amaral, M.E., Anhê, G.F., Delghingaro-Augusto, V., Cunha, D.A., Nicoletti-Carvalho, J.E., Boschero, A.C., 2004. Prolactin-modulated gene expression profiles in pancreatic islets from adult female rats. Mol. Cell Endocrinol. 220, 41–50.

Borge, B.A., Kalland, K.H., Olsen, S., Bletsa, A., Berggreen, E., Wiig, H., 2009. Cytokines are produced locally by myocytes in rat skeletal muscle during endotoxemia. Am. J. Physiol. Heart Circ. Physiol. 296, H735–744.

Borst, S.E., Conover, C.F., 2005. High-fat diet induces increased tissue expression of TNF-alpha. Life Sci. 77, 2156–2165.

Carvalho-Filho, M.A., Ueno, M., Hirabara, S.M., Seabra, A.B., Carvalheira, J.B., de Oliveira, M.G., Velloso, L.A., Curi, R., Saad, M.J., 2005. S-nitrosation of the insulin receptor, insulin receptor substrate 1, and protein kinase B/Akt: a novel mechanism of insulin resistance. Diabetes 54, 959–967.

Chiang, S.H., Bazuine, M., Lumeng, C.N., Geletka, L.M., Mowers, J., White, N.M., Ma, J.T., Zhou, J., Qi, N., Westcott, D., Delproposto, J.B., Blackwell, T.S., Yull, F.E., Saltiel, A.R., 2009. The protein kinase IKKepsilon regulates energy balance in obese mice. Cell 138, 961–975.

Coskun, O., Kanter, M., Korkmaz, A., Oter, S., 2005. Quercetin, a flavonoid antioxidant, prevents and protects streptozotocin-induced oxidative stress and beta-cell damage in rat pancreas. Pharmacol. Res. 51, 117–123.

Crozier, A., Lean, M.E., Donald, M.S., Black, C., 1997. Quantitative analysis of the flavonoid content of commercial tomatoes, onions, lettuce, and celery. J. Agric. Food Chem. 45, 590–595.

DeFronzo, R.A., 2004. Pathogenesis of type 2 diabetes mellitus. Med. Clin. North Am. 88, 787–835.

Dias, A.S., Porawski, M., Alonso, M., Marroni, N., Collado, P.S., González-Gallego, J., 2005. Quercetin decreases oxidative stress, NF-kappaB activation, and iNOS overexpression in liver of streptozotocin-induced diabetic rats. J. Nutr. 135, 2299–2304.

Fang, X.K., Gao, J., Zhu, D.N., 2008. Kaempferol and quercetin isolated from *Euonymus alatus* improve glucose uptake of 3T3-L1 cells without adipogenesis activity. Life Sci. 82, 615–622.

Fernández-Real, J.M., Ricart, W., 2002. Insulin resistance and chronic cardiovascular inflammatory syndrome. Endocr. Rev. 24, 278–301.

Furuya, D.T., Poletto, A.C., Favaro, R.R., Martins, J.O., Zorn, T.M., Machado, U.F., 2010. Anti-inflammatory effect of atorvastatin ameliorates insulin resistance in monosodium glutamate-treated obese mice. Metabolism 59, 395–399.

Gao, Z., Yin, J., Zhang, J., He, Q., McGuinness, O.P., Ye, J., 2009. Inactivation of NF-kappaB p50 leads to insulin sensitization in liver through post-translational inhibition of p70S6K. J. Biol. Chem. 284, 18368–18376.

Gilmore, T.D., 1999. The Rel/NF-kappaB signal transduction pathway: introduction. Oncogene 18, 6842–6844.

Granado-Serrano, A.B., Martín, M.A., Bravo, L., Goya, L., Ramos, S., 2006. Quercetin induces apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI-3-kinase/Akt and ERK pathways in a human hepatoma cell line (HepG2). J. Nutr. 136, 2715–2721.

Groop, L., Saloranta, C., Shank, M., Bonadonna, R.C., Ferrannini, E., DeFronzo, R.A., 1991. The role of free fatty acid metabolism in the pathogenesis of insulin resistance in obesity and non-insulin dependent diabetes mellitus. J. Clin. Endocrinol Metab. 72, 96–107.

Hirosumi, J., Tuncman, G., Chang, L., Görgün, C.Z., Uysal, K.T., Maeda, K., Karin, M., Hotamisligil, G.S., 2002. A central role for JNK in obesity and insulin resistance. Nature 420, 333–336.

Hu, J., Huang, Y., Xiong, M., Luo, S., Chen, Y., Li, Y., 2006. The effects of natural flavonoids on lipoxygenase-mediated oxidation of compounds with a benzene

ring structure—a new possible mechanism of flavonoid anti-chemical carcinogenesis and other toxicities. Int. J. Toxicol. 25, 295–301.
Jové, M., Planavila, A., Sánchez, R.M., Merlos, M., Laguna, J.C., Vázquez-Carrera, M., 2006. Palmitate induces tumor necrosis factor-alpha expression in C2C12 skeletal muscle cells by a mechanism involving protein kinase C and nuclear factor-kappaB activation. Endocrinology 147, 552–561.
Katsuki, A., Sumida, Y., Murashima, S., Murata, K., Takarada, Y., Ito, K., Fujii, M., Tsuchihashi, K., Goto, H., Nakatani, K., Yano, Y., 1998. Serum levels of tumor necrosis factor-alpha are increased in obese patients with noninsulin-dependent diabetes mellitus. J. Clin. Endocrinol. Metab. 83, 859–862.
Kim, E.K., Kwon, K.B., Song, M.Y., Han, M.J., Lee, J.H., Lee, Y.R., Lee, J.H., Ryu, D.G., Park, B.H., Park, J.W., 2007. Flavonoids protect against cytokine-induced pancreatic beta-cell damage through suppression of nuclear factor kappaB activation. Pancreas 35, e1–9.
Kundu, J.K., Surh, Y.J., 2004. Molecular basis of chemoprevention by resveratrol: NF-kappaB and AP-1 as potential targets. Mutat. Res. 555, 65–80.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.
Nait Chabane, M., Al Ahmad, A., Peluso, J., Muller, C.D., Ubeaud, G., 2009. Quercetin and naringenin transport across human intestinal Caco-2 cells. J. Pharm Pharmacol. 61, 1473–1483.
Plomgaard, P., Bouzakri, K., Krogh-Madsen, R., Mittendorfer, B., Zierath, J.R., Pedersen, B.K., 2005. Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. Diabetes 54, 2939–2945.
Rechner, A.R., Kuhnle, G., Hu, H., Roedig-Penman, A., van den Braak, M.H., Moore, K.P., Rice-Evans, C.A., 2002. The metabolism of dietary polyphenols and the relevance to circulating levels of conjugated metabolites. Free Radic. Res. 36, 1229–1241.
Rivera, L., Morón, R., Sánchez, M., Zarzuelo, A., Galisteo, M., 2008. Quercetin ameliorates metabolic syndrome and improves the inflammatory status in obese Zucker rats. Obesity (Silver Spring) 16, 2081–2087.
Romero, M., Jiménez, R., Hurtado, B., Moreno, J.M., Rodríguez-Gómez, I., López-Sepúlveda, R., Zarzuelo, A., Pérez-Vizcaino, F., Tamargo, J., Vargas, F., Duarte, J., 2010. Lack of beneficial metabolic effects of quercetin in adult spontaneously hypertensive rats. Eur. J. Pharmacol. 627, 242–250.
Ruan, H., Hacohen, N., Golub, T.R., Van Parijs, L., Lodish, H.F., 2002. Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory. Diabetes 51, 1319–1336.
Sabin, M.A., Stewart, C.E., Crowne, E.C., Turner, S.J., Hunt, L.P., Welsh, G.I., Grohmann, M.J., Holly, J.M., Shield, J.P., 2007. Fatty acid-induced defects in insulin signalling, in myotubes derived from children, are related to ceramide production from palmitate rather than the accumulation of intramyocellular lipid. J. Cell Physiol. 211, 244–252.
Senn, J.J., 2006. Toll-like receptor-2 is essential for the development of palmitate-induced insulin resistance in myotubes. J. Biol. Chem. 281, 26865–26875.
Senthilkumar, K., Elumalai, P., Arunkumar, R., Banudevi, S., Gunadharini, N.D., Sharmila, G., Selvakumar, K., Arunakaran, J., 2010. Quercetin regulates insulin like growth factor signaling and induces intrinsic and extrinsic pathway mediated apoptosis in androgen independent prostate cancer cells (PC-3). Mol. Cell Biochem. 344, 173–184.
Silva, J.L., Giannocco, G., Furuya, D.T., Lima, G.A., Moraes, P.A., Nachef, S., Bordin, S., Britto, L.R., Nunes, M.T., Machado, U.F., 2005. NF-kappaB, MEF2A, MEF2D and HIF1-a involvement on insulin- and contraction-induced regulation of GLUT4 gene expression in soleus muscle. Mol. Cell Endocrinol. 240, 82–93.
Steinberg, G.R., Michell, B.J., van Denderen, B.J., Watt, M.J., Carey, A.L., Fam, B.C., Andrikopoulos, S., Proietto, J., Görgün, C.Z., Carling, D., Hotamisligil, G.S., Febbraio, M.A., Kay, T.W., Kemp, B.E., 2006. Tumor necrosis factor alpha-induced skeletal muscle insulin resistance involves suppression of AMP-kinase signaling. Cell Metab. 4, 465–474.
Stewart, L.K., Wang, Z., Ribnicky, D., Soileau, J.L., Cefalu, W.T., Gettys, T.W., 2009. Failure of dietary quercetin to alter the temporal progression of insulin resistance among tissues of C57BL/6 J mice during the development of diet-induced obesity. Diabetologia 52, 514–523.
Taniguchi, C.M., Emanuelli, B., Kahn, C.R., 2006. Critical nodes in signalling pathways: insights into insulin action. Nat. Rev. Mol. Cell Biol. 7, 85–96.
Thrush, A.B., Heigenhauser, G.J., Mullen, K.L., Wright, D.C., Dyck, D.J., 2008. Palmitate acutely induces insulin resistance in isolated muscle from obese but not lean humans. Am J. Physiol. Regul. Integr. Comp. Physiol. 294, R1205–1212.
Tilg, H., Moschen, A.R., 2008. Inflammatory mechanisms in the regulation of insulin resistance. Mol. Med. 14, 222–231.
Uysal, K.T., Wiesbrock, S.M., Marino, M.W., Hotamisligil, G.S., 1997. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature 389, 610–614.
Vessal, M., Hemmati, M., Vasei, M., 2003. Antidiabetic effects of quercetin in streptozocin-induced diabetic rats. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 135C, 357–364.
Wallberg-Henriksson, H., Zierath, J.R., 2001. GLUT4: a key player regulating glucose homeostasis? Insights from transgenic and knockout mice (review). Mol. Membr. Biol. 18, 205–211.
Ying, B., Yang, T., Song, X., Hu, X., Fan, H., Lu, X., Chen, L., Cheng, D., Wang, T., Liu, D., Xu, D., Wei, Y., Wen, F., 2009. Quercetin inhibits IL-1 beta-induced ICAM-1 expression in pulmonary epithelial cell line A549 through the MAPK pathways. Mol. Biol. Rep. 36, 1825–1832.