

Long-Term Pioglitazone Treatment Augments Insulin Sensitivity and PKC- ϵ and PKC- θ Activation in Skeletal Muscles in Sucrose Fed Rats

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Summary

It has been suggested that thiazolidinediones (TZDs) ameliorate insulin resistance in muscle tissue by suppressing muscle lipid storage and the activity of novel protein kinase C (nPKC) isoforms. To test this hypothesis, we analyzed long-term metabolic effects of pioglitazone and the activation of nPKC- ϵ and - θ isoforms in an animal model of the metabolic syndrome, the spontaneously hypertensive rat (a congenic SHR strain with wild type *Cd36* gene) fed a diet with 60 % sucrose from the age of 4 to 8 months. Compared to untreated controls, pioglitazone treatment was associated with significantly increased basal (809 ± 36 vs 527 ± 47 nmol glucose/g/2h, $P < 0.005$) and insulin-stimulated glycogenesis (1321 ± 62 vs 749 ± 60 nmol glucose/g/2h, $P < 0.0001$) in isolated gastrocnemius muscles despite increased concentrations of muscle triglycerides (3.83 ± 0.33 vs 2.25 ± 0.12 $\mu\text{mol/g}$, $P < 0.005$). Pioglitazone-treated rats exhibited significantly increased membrane/total (cytosolic plus membrane) ratio of both PKC- ϵ and PKC- θ isoforms compared to untreated controls. These results suggest that amelioration of insulin resistance after long-term pioglitazone treatment is associated with increased activation of PKC- ϵ and - θ isoforms in spite of increased lipid concentration in skeletal muscles.

Key words

Novel PKC • Pioglitazone • Muscle lipids • Insulin resistance • Rat

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Introduction

Thiazolidinediones (TZDs) such as pioglitazone are insulin sensitizing drugs used for the treatment of type 2 diabetes. TZDs improve glycemic control by reducing insulin resistance in target tissues, however, their mechanism of action is not fully understood (Zinn *et al.* 2008). In skeletal muscles, insulin resistance is often associated with accumulation of intramyocellular lipids. For instance, strong negative correlations between intramyocellular lipids and insulin sensitivity were found in obese nondiabetic and type 2 diabetic subjects (Philips *et al.* 1996, Forouhi *et al.* 1999, Perseghin *et al.* 1999, Kelley *et al.* 2002) and in animal models of obesity and type 2 diabetes (Divišová *et al.* 2002, Pravenec *et al.* 2003, Qi *et al.* 2005). TZDs were reported to reduce intramyocellular lipids in most studies (Hockings *et al.* 2003, Jucker *et al.* 2003, Kim *et al.* 2003, Koh *et al.* 2003, Kuhlmann *et al.* 2003) and accordingly it is possible that intramyocellular lipid reduction might represent the molecular mechanism of TZD-mediated insulin-sensitizing effects in skeletal muscles. Intramyocellular triglycerides are regarded only as a marker of other lipid intermediates such as diacylglycerols or ceramides that could interfere with insulin signaling. Diacylglycerols activate protein kinase C (PKC) isoforms that can inhibit several steps of the insulin signaling cascade and in addition also downstream metabolic enzymes such as glycogen synthase (Schmitz-Peiffer *et al.* 2002, 2008). PKCs represent a family of proteins with distinct structural and functional features and specifically the novel isoforms nPKC- δ , - ϵ , - θ and -

η have been implicated in the pathogenesis of insulin resistance and their modulation by TZDs might be important for insulin-sensitizing effects. Especially PKC- θ is highly expressed in skeletal muscles compared to other tissues and is thus considered as an important candidate for regulating glucose homeostasis. In the current study, we tested a hypothesis that TZDs ameliorate insulin resistance in muscle tissue by suppressing muscle lipid storage and the activation of nPKC isoenzymes. Specifically, we analyzed long-term metabolic effects of pioglitazone in an animal model of the metabolic syndrome, the spontaneously hypertensive rat (congenic SHR strain with wild type *Cd36* gene), fed a diet with 60 % sucrose from the age of 4 to 8 months. The SHR congenic strain of with wild type *Cd36* gene was selected instead of the SHR that harbors a deletion variant because *Cd36* is a target gene involved in the insulin-sensitizing actions of pioglitazone (Qi *et al.* 2002).

Methods

Animals

The SHR.BN-*Il6/Npy* (hereafter referred to as SHR-4) congenic strain was derived by transferring a segment of chromosome 4 carrying wild type *Cd36* from inbred Brown Norway strain (Charles River Laboratories) (BN/Crl) onto the SHR/Ola genetic background. The SHR-4 congenic strain is genetically identical to the SHR progenitor strain except for the differential segment of chromosome 4 including *Cd36* (Pravenec *et al.* 1999). SHR-4 congenic rats of the N8F18 generation were used in the current study. The SHR-4 congenic strain with wild type *Cd36* was used instead of the SHR strain with a deletion variant of *Cd36* because *Cd36* is a target gene involved in the insulin-sensitizing actions of a TZD ligand of PPAR γ (Qi *et al.* 2002). The rats were housed in an air-conditioned animal facility and allowed free access to food and water. We fed male SHR-4 controls (n=9) a diet containing 60 % sucrose from the age of 4 months for 120 days. SHR-4 males in the experimental group (n=9) were fed the same diet supplemented with pioglitazone (300 mg/kg diet). At the end of the study, rats were killed by decapitation in *ad libitum*-fed state. All experiments were performed in agreement with the Animal Protection Law of the Czech Republic (311/1997) and were approved by the Ethics Committee of the Institute of Physiology, Czech Academy of Sciences and by the Ethics Committee of the Czech Academy of Sciences, Prague.

Biochemical analyses

Blood glucose levels were measured by the glucose oxidase assay (Pliva-Lachema, Brno, Czech Republic) using tail vein blood drawn into 5 % trichloroacetic acid and promptly centrifuged. Serum triglyceride concentrations were measured by standard enzymatic methods (Pliva-Lachema, Brno, Czech Republic). Serum insulin concentrations were determined using Mercodia Rat Insulin ELISA kit (Mercodia AB, Uppsala, Sweden).

Western blot analysis

Muscle tissue was homogenized and processed to obtain cytosolic and membrane fraction as described previously (Neckář *et al.* 2005). Briefly, frozen samples of gastrocnemius muscle were homogenized and centrifuged at 105,000 g for 60 min. The supernatant was removed as the cytosolic fraction, the sediment (membrane fraction) was resuspended in homogenization buffer containing 1 % Triton X-100, held on ice for 60 min and then centrifuged at 105,000 g for further 60 min. Aliquots of both fractions were saved for protein quantification (Lowry assay modified by Peterson), and cytosol and membrane samples were then used for Western blotting. Samples were run on sodium dodecylsulphate polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). The membranes were then incubated in 5 % dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS, pH 7.5) for 60 min at room temperature. After the blocking step, membranes were washed in TTBS buffer and incubated with PKC- ϵ and PKC- θ -specific polyclonal primary rabbit antisera (PKC- ϵ : Sigma, Saint Luis, MO, USA; PKC- θ : Santa Cruz Biotechnology, Santa Cruz, CA, USA). After further washings, membranes were incubated with the secondary swine anti-rabbit IgG antibody labeled with horseradish peroxidase (Sevapharma, Prague, Czech Republic). Immunoreactive proteins were made visible using chemiluminescence reagents (Amersham Int., Aurora, Ohio, USA) and exposure to autoradiographic film. Scanning and Quantity One softwares (Bio-Rad, Hercules, CA, USA) were used for quantification of the relative abundance of individual PKC isoforms. To ensure the specificity of PKC- ϵ and PKC- θ immunoreactive proteins, prestained molecular weight protein standards (Fluka, Buchs, Switzerland), recombinant human PKC- ϵ and PKC- θ standards

(Sigma), rat brain extracts, and the respective competing immunizing peptides were used.

Skeletal muscle glycogen synthesis

Glycogen synthesis was determined in isolated gastrocnemius muscle by measuring the incorporation of ^{14}C -U glucose into glycogen as previously described (Qi *et al.* 2005). The gastrocnemius muscles were attached to a stainless steel frame *in situ* at *in vivo* length by special clips and separated from other muscles and tendons and immediately incubated for 2 h in Krebs-Ringer bicarbonate buffer, pH 7.4 that contained 5.5 mM unlabeled glucose, 0.5 $\mu\text{Ci/ml}$ of ^{14}C -U glucose, and 3 mg/ml bovine serum albumin (Armour, Fraction V) with or without 250 $\mu\text{U/ml}$ insulin. Glycogen was extracted and glucose incorporation into glycogen determined as previously described (Qi *et al.* 2005).

Tissue triglyceride measurements

For triglyceride determination, gastrocnemius muscles were powdered under liquid N_2 and extracted for 16 h in chloroform:methanol, after which 2 % KH_2PO_4 was added, and the solution was centrifuged. The organic phase was removed and evaporated under N_2 . The resulting pellet was dissolved in isopropyl alcohol, and triglyceride content was determined by enzymatic assay (Pliva-Lachema, Brno, Czech Republic).

Statistical analysis

All data are expressed as means \pm S.E.M. Differences between control and experimental groups were evaluated by paired or non-paired t tests as appropriate. Statistical significance was defined as $p < 0.05$.

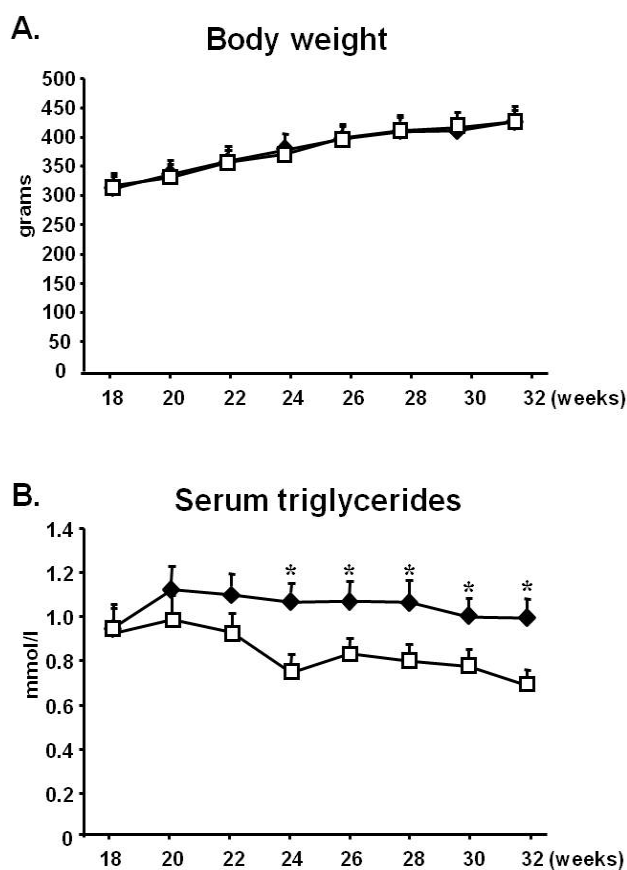


Fig. 1. Effects of long-term pioglitazone treatment on body weight and serum triglyceride levels in SHR-4 rats fed a high-sucrose diet. **A.** Body weights in control rats (solid diamonds) were similar to rats treated with pioglitazone (open squares). **B.** Serum triglyceride levels were significantly decreased after 6 weeks of pioglitazone treatment compared to control untreated rats (symbols as above). * denotes $P < 0.01$.

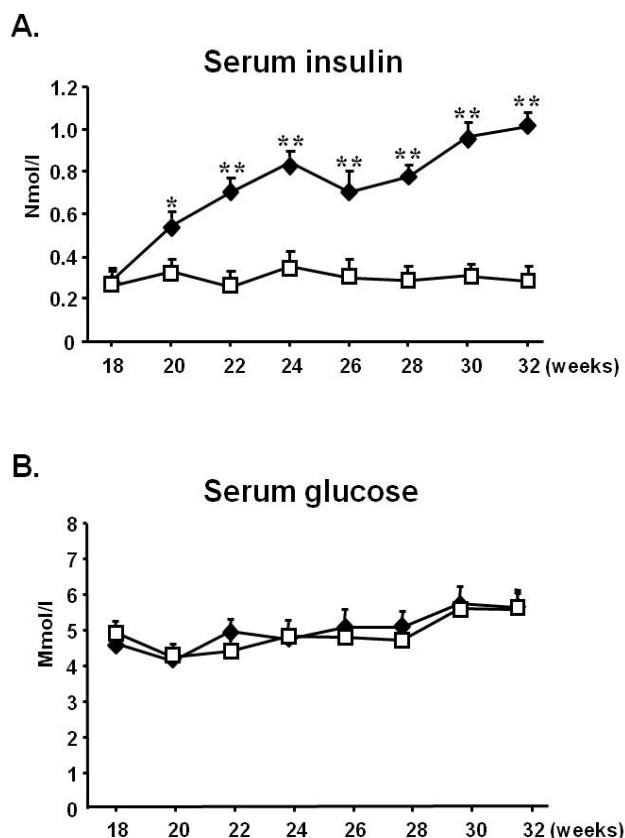


Fig. 2. Effects of long-term pioglitazone treatment on serum insulin and glucose levels in SHR-4 rats fed a high-sucrose diet. **A.** Serum insulin levels were significantly decreased after two weeks pioglitazone treatment (open squares) when compared to untreated controls (solid diamonds). **B.** There were no significant differences in blood glucose levels between pioglitazone treated and control rats (symbols as above). * and ** denote $P < 0.0001$ and $P < 0.00001$, respectively.

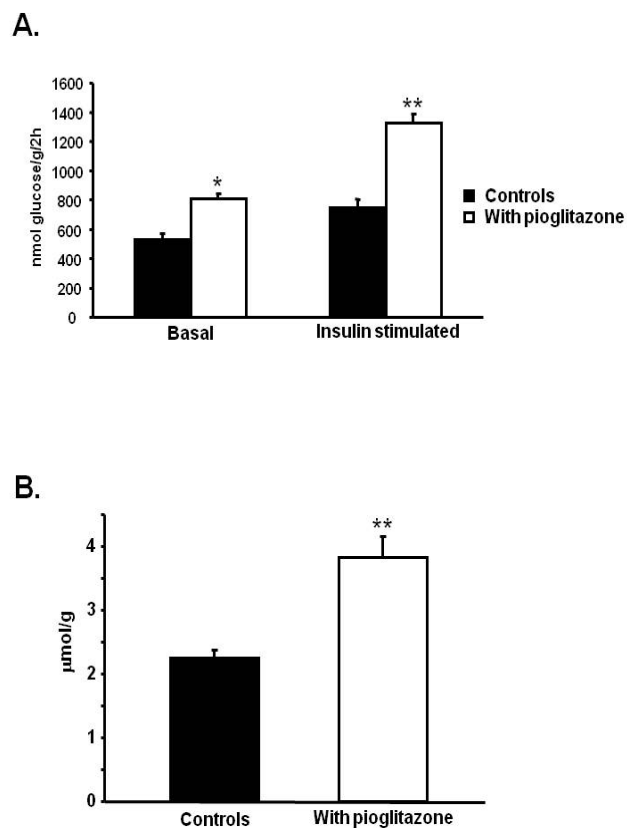


Fig. 3. Effects of pioglitazone treatment on gastrocnemius muscle sensitivity to insulin action and on triglyceride concentration in SHR-4 rats fed a high-sucrose diet. **A.** Effects of pioglitazone treatment on glycogenesis in isolated gastrocnemius muscles. Basal and insulin-stimulated incorporation of radioactively labeled glucose into muscle glycogen was significantly greater in pioglitazone-treated rats (open bars) when compared to controls (solid bars). **B.** Triglyceride concentrations in isolated gastrocnemius muscles were significantly greater in pioglitazone treated rats (open bars) when compared to controls (solid bars). * and ** denote $P < 0.005$ and $P < 0.0001$, respectively.

Results

Body weights in pioglitazone-treated and control rats were similar (Fig. 1A). After 6 weeks of high-sucrose diet feeding, pioglitazone-treated rats exhibited significantly decreased levels of serum triglycerides (Fig. 1B). High sucrose feeding was associated with a progressive increase in serum insulin levels in control rats, while no hyperinsulinemia was observed in pioglitazone-treated rats (Fig. 2A). Both pioglitazone-treated and control rats showed similar normal levels of blood glucose (Fig. 2B). Pioglitazone-treated rats exhibited significant amelioration of insulin resistance in skeletal muscles as estimated from increased basal and insulin-stimulated glycogenesis (Fig. 3A) despite the fact that pioglitazone treatment was associated with increased concentrations of muscle triglycerides (Fig. 3B).

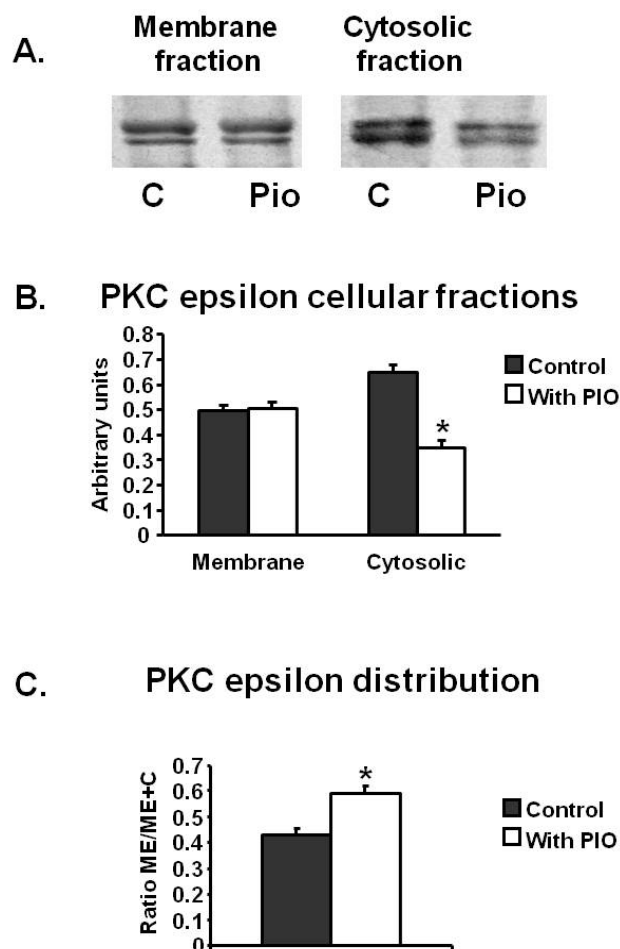


Fig. 4. The effects of pioglitazone treatment on PKC- ϵ activation in gastrocnemius muscle isolated from SHR-4 rats fed a high-sucrose diet. **A** and **B.** Western blot analysis of PKC- ϵ membrane and cytosolic fractions in controls (solid bars) and pioglitazone-treated rats (open bars). There was a significant decrease in the expression of cytosolic fraction in pioglitazone-treated rats. **C.** Ratio of membrane to total fractions representing the activation of PKC- ϵ . Pioglitazone treatment was associated with a significant increase in PKC- ϵ activation. * denotes $P < 0.01$.

Pioglitazone treatment was associated with significantly increased ratio of membrane to total PKC- ϵ and PKC- θ proteins (Figs 4 and 5) suggesting activation of both nPKC isoforms.

Discussion

In the current study, a long-term pioglitazone treatment of rats fed a high-sucrose diet was associated with amelioration of insulin resistance in gastrocnemius muscles despite the fact that muscle triglyceride concentrations were significantly increased. In addition, amelioration of insulin resistance in skeletal muscles was associated with a significant activation of both PKC- ϵ and PKC- θ as reflected by increased membrane/total ratio of both nPKC isoforms. Contrary to these results, it has

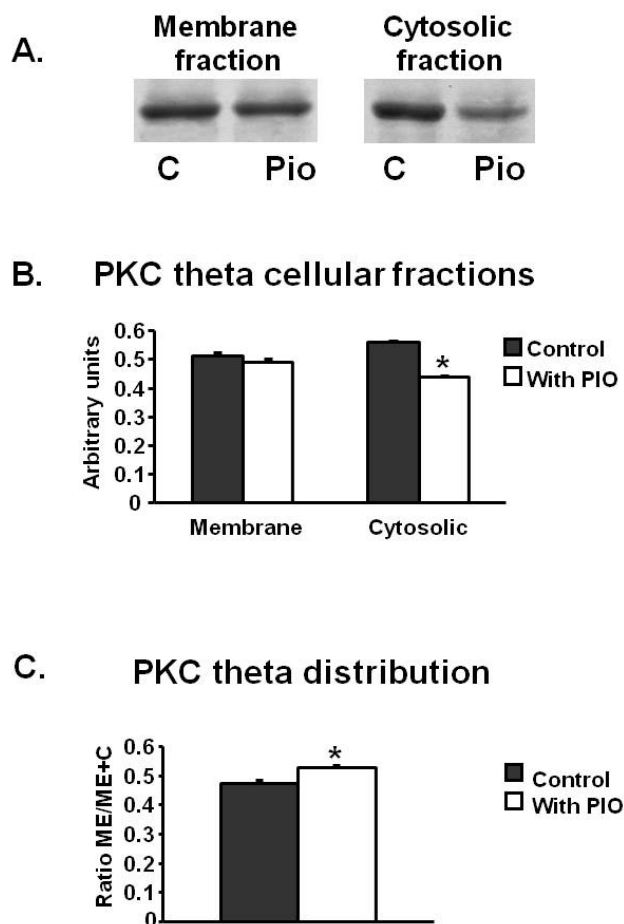


Fig. 5. The effects of pioglitazone treatment on PKC- θ activation in gastrocnemius muscle isolated from SHR-4 rats fed a high-sucrose diet. **A** and **B.** Western blot analysis of PKC- θ membrane and cytosolic fractions in controls (solid bars) and pioglitazone-treated rats (open bars). There was a significant decrease in the expression of cytosolic fraction in pioglitazone-treated rats. **C.** Ratio of membrane to total fractions representing the activation of PKC- θ . Pioglitazone treatment was associated with a significant increase in PKC- θ activation. * denotes $P < 0.01$.

been reported that insulin resistance induced by feeding high-fat diets was associated with increased activities of nPKC isoforms suggesting that activation of nPKC isoforms might inhibit insulin signaling and induce insulin resistance (Schmitz-Peiffer *et al.* 1997, Yu *et al.* 2002). On the other hand, studies in PKC- θ knockout mice demonstrated that the absence of functional PKC- θ predisposes to obesity and insulin resistance when mice were fed a high-fat diet (Gao *et al.* 2007). Also transgenic mice with a dominant negative PKC- θ in skeletal muscle exhibited obesity and insulin resistance (Serra *et al.* 2003). In addition, PKC- ϵ gene knockout mice showed increased serum insulin during i.p. GTT suggesting insulin resistance as compared to wild type controls (Schmitz-Peiffer *et al.* 2007). These findings are consistent with results of the current study when

increased insulin sensitivity in gastrocnemius muscles isolated from pioglitazone-treated rats was associated with PKC- θ and PKC- ϵ activation and provide evidence that nPKC isoforms are directly involved in regulating muscle sensitivity to insulin action.

Schmitz-Peiffer *et al.* (1997) reported that a 4-day treatment of Wistar rats fed a high-fat diet with a TZD BRL-49653 was associated with significantly decreased membrane/cytosolic ratio suggesting reduced functional activation of PKC- θ isoforms in BRL-49653 treated rats. These results are opposite to those reported in the current study when pioglitazone-treated rats exhibited increased membrane/cytosolic ratio. In addition, Lessard *et al.* (2004) found no significant changes in total or membrane-associated PKC- θ fraction in soleus muscle isolated from obese Zucker rats fed a standard rat chow and treated for 4 weeks with rosiglitazone when compared to either lean or obese controls. These discrepant results could be explained by studying different types of muscles, different TZDs, different animal models as well as different diets.

TZD treatment is usually associated with significantly decreased intramyocellular lipid levels (Philips *et al.* 1996, Forouhi *et al.* 1999, Perseghin *et al.* 1999, Divišová *et al.* 2002, Kelley *et al.* 2002, Pravenec *et al.* 2003, Qi *et al.* 2005). According to a widely accepted hypothesis, lipid reducing effects of TZD in skeletal muscles are considered as possible insulin-sensitizing mechanisms acting through reducing the activity of especially novel PKC isoforms thereby ameliorating insulin signaling. Surprisingly, in the current study, we observed that long-term pioglitazone treatment was associated with increased triglyceride levels in gastrocnemius muscle, with activation of the PKC- ϵ and PKC- θ isoforms, and with amelioration of insulin-stimulated glycogenesis. In addition, pioglitazone-treated rats exhibited significantly decreased levels of serum insulin and triglycerides suggesting amelioration of systemic insulin resistance and dyslipidemia when fed a high-sucrose diet. These findings are similar to results reported by Lessard *et al.* (2004) who found ameliorated glucose tolerance in obese Zucker rats treated with rosiglitazone despite the fact that these rats exhibited significantly increased intramyocellular triglycerides in soleus muscles when compared to glucose-intolerant controls. The reason for discrepant results regarding TZD effects on skeletal muscle lipid accumulation is unclear. It has been suggested that the effects of TZD on lipid storage might depend on the predominant type of muscle

fibers. For instance, most of studies showing reduced skeletal muscle lipid content after TZD treatment analyzed muscles with a large percentage of type II fibers, while Lessard *et al.* (2004) analyzed soleus muscle containing predominantly type I fibers. Gastrocnemius muscle used in the current study, contains both type I and type II fibers, suggesting that differential effects of TZD on lipid storage in skeletal muscle might not depend on the muscle type. It is also possible that increased triglyceride levels in gastrocnemius muscle might be explained by reduced activity of hormone-sensitive lipase (HSL) that is a lipase for diacylglycerols (DAG) (Heammerle *et al.* 2002). Downregulation of HSL could be associated with increased DAG which might affect activation of PKC. Rosiglitazone treatment was associated with 65 % increase in DAG (Lessard *et al.* 2004). Both PKC- θ and PKC- ϵ analyzed in the current study are DAG-sensitive.

In conclusion, results of the current study demonstrate that long-term pioglitazone treatment can

ameliorate insulin resistance in muscle tissue by mechanisms that might include activation of PKC- ϵ and PKC- θ isoforms and that are independent on reduction in muscle lipid content.

Conflict of Interest

There is no conflict of interest.

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