Rosiglitazone, an agonist of peroxisome-proliferator-activated receptor γ (PPARγ), decreases inhibitory serine phosphorylation of IRS1 *in vitro* and *in vivo*

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Peroxisome-proliferator-activated receptor γ agonists such as rosiglitazone, a thiazolidinedione, improve insulin sensitivity *in vivo*, but the underlying mechanism(s) remains unclear. Phosphorylation of IRS1 (insulin receptor substrate protein 1) on certain serine residues, including S307 and S612 in rodent IRS1 (equivalent to S312 and S616 in human IRS1), has been shown to play a negative role in insulin signalling. In the present study, we investigated whether rosiglitazone improves insulin sensitivity by decreasing IRS1 inhibitory serine phosphorylation. In HEK-293 (human embryonic kidney 293) cells stably expressing recombinant IRS1 and in 3T3L1 adipocytes, rosiglitazone attenuated PMA-induced IRS1 S307/S612 phosphorylation and decreased insulin-stimulated Akt phosphorylation. We observed increased IRS1 S307 phosphorylation and concomitant decrease in insulin signalling as measured by insulin-stimulated IRS1 tyrosine phosphorylation, and Akt threonine phosphorylation in adipose tissues of Zucker obese rats compared with lean control rats. Treatment with rosiglitazone at 30 mg/kg body weight for 24 and 48 h increased insulin signalling and decreased IRS1 S307 phosphorylation concomitantly. Whereas the 48 h treatment reversed hyper-phosphorylation (and activation) of both c-Jun N-terminal kinase and p38 mitogen-activated protein kinase, the 24 h treatments only decreased hyper-phosphorylation of p38 mitogen-activated protein kinase. The treatment of the Zucker obese rats with rosiglitazone also reversed the high circulating levels of non-esterified fatty acids, which have been shown to be correlated with increased IRS1 serine phosphorylation in other animal models. Taken together, these results suggest that IRS1 inhibitory serine phosphorylation is a key component of insulin resistance and its reversal contributes to the insulin sensitizing effects by rosiglitazone.

Key words: insulin receptor substrate 1, insulin sensitization, peroxisome-proliferator-activated receptor, rosiglitazone, serine phosphorylation.

INTRODUCTION

TZDs (thiazolidinediones, e.g. rosiglitazone, troglitazone and pioglitazone) are a class of antidiabetic drugs that improve insulin sensitivity *in vivo* [1,2]. The physiological effects of TZDs are well documented. For instance, they decrease the levels of circulating insulin, non-esterified fatty acids and triacylglycerols, and increase insulin-stimulated glucose uptake and utilization in both animal models and human subjects [1,2]. TZDs are ligands of PPARγ (peroxisome-proliferator-activated receptor γ), a member of a larger family of the ligand-activated nuclear receptor transcription factors. Whereas it is generally thought that binding and activation of PPARγ is the predominant mechanism by which TZDs mediate their antidiabetic efficacy, the precise molecular events downstream of PPARγ activation that are critical for TZDs-mediated insulin sensitization *in vivo* are less clear [3].

The action of insulin is mediated via the IR (insulin receptor). IR is a heterotetramer consisting of two extracellular α-subunits (IRα) and two transmembrane β-subunits (IRβ) with intrinsic protein tyrosine kinase activity. The binding of the insulin to IRα subunit leads to autophosphorylation and activation of the IRβ subunit. Cytoplasmic signalling molecules including IRS1–4 (IR substrate proteins 1–4) bind to the phosphotyrosine residues in the activated IRβ subunits and subsequently become tyrosine phosphorylated by IRβ subunits. The phosphotyrosine residues in the IRS proteins provide docking sites for further downstream signalling molecules including the p85 subunit of the PI3K (phosphoinositide 3-kinase). This binding leads to activation of the p110 catalytic subunit of the PI3K, a key mediator required for insulin-induced physiological effects. Activated PI3K catalyses the production of phosphatidylinositide 3,4-biphosphate and phosphatidylinositide 3,4,5-triphosphate, which in turn bind to and activate further downstream kinases including the phosphoinositide-dependent serine/threonine kinase 1, Akt and PKCλ/ζ (protein kinase C), all of which are known to play key roles in insulin-induced metabolic effects [4,5].

There is emerging evidence that TZDs exert their insulin-sensitizing effects at least partially by potentiating insulin signalling. Insulin-stimulated PI3K and Akt activation is blunted in diabetic and insulin-resistant states in animals and human beings [6–10]. Treatments with TZDs potentiate insulin-stimulated PI3K and Akt activation in cultured cells *in vitro* [10–13]. Long-term treatment with rosiglitazone increases insulin-stimulated Akt phosphorylation in the skeletal muscle of normoglycaemic human beings at risk for Type II diabetes [14]. Long-term treatment with rosiglitazone, particularly in combination with exercise, also increased insulin-stimulated IRβ subunit tyrosine phosphorylation and Akt serine phosphorylation in Zucker obese rats [8]. We reported previously that treatments with PPARγ agonists including rosiglitazone acutely normalize impaired insulin signalling

Abbreviations used: ECF, enhanced chemifluorescence; ECL®, enhanced chemiluminescence; IR, insulin receptor; IRS1–4, IR substrate proteins 1–4; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; mpk, mg/kg body weight; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PPARγ, peroxisome-proliferator-activated receptor γ; TZD, thiazolidinedione; upk, units/kg body weight; for brevity the one-letter system for amino acids has been used; S307, e.g., means Ser^{307}.

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in the Zucker obese rats by increasing the insulin-stimulated phosphorylation of IRβ subunits, IRS1, Akt and glycogen synthase kinase 3 in both adipose and muscle tissues [10].

The studies discussed above indicate that potentiation of insulin signalling is probably a part of the mechanisms underlying PPARγ agonist-mediated insulin sensitization *in vivo*. The detailed mechanisms at the signalling molecule level warrant further study. In addition to tyrosine phosphorylation, the IR and IRS proteins undergo serine phosphorylation that attenuates insulin signalling [15–19]. The inhibitory phosphorylation of IRS1 is known to occur on multiple serine residues, including S307 and S612 of rodent IRS1 (equivalent to S312 and S616 of human IRS1; for convenience, S307 and S612 are used throughout the paper for both human and rodent IRS1) [16,17,20–23]. To date, several serine/threonine kinases have been implicated in this process; these include PKC isoforms (i.e. PKCθ) [24,25], the inhibitor of nuclear factor-κB kinase β [26–29] and JNK (c-Jun N-terminal kinase) [17,30,31]. These inhibitory serine phosphorylation events function as negative feedback loops for insulin signal transduction and provide a basis for cross-talk with other pathways through which insulin resistance may be mediated [32]. Indeed, increase in IRS1 serine phosphorylation and/or activation of serine kinases have been observed in tissues of obese animals [31], animals infused with non-esterified fatty acids [25], and in primary adipocytes of Type II diabetic human subjects [33].

To further our understanding of the mechanism underlying PPARγ agonists-mediated insulin sensitization, the present study was initiated to investigate whether increased IRS1 inhibitory serine phosphorylation occurs under insulin-resistant states and, if so, whether such inhibitory phosphorylation can be reversed by treatments with rosiglitazone *in vitro* as well as *in vivo*.

MATERIALS AND METHODS

Materials

Insulin and PMA were purchased from Sigma. Rabbit polyclonal antibodies against total IRS1 (IRS1-total) and IRS1-pS307 (IRS1 phosphorylated at S307) and monoclonal antibody against tyrosine-phosphorylated proteins (clone 4G10) were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Rabbit polyclonal antibodies against IRS1, phosphorylated at S612, were purchased from BioSource International (Camarillo, CA, U.S.A.). Rabbit polyclonal antibodies against total Akt (Akt-total), and Akt-pT308 or Akt-pS473 (Akt phosphorylated at T308 or S473), total p38 (p38-total) MAPK (mitogen-activated protein kinase), p38-pT180/Y182 (p38 MAPK phosphorylated at T180 and T182), total p42/44 MAPK (p42/44-total), p42/44-pT202/Y204 (p42/44 MAPK phosphorylated at T202 and T204), total JNK (JNK-total) and JNK-pT183/Y185 (JNK phosphorylated at T183 and T185) were purchased from Cell Signaling (Beverly, MA, U.S.A.).

Cell culture, rosiglitazone treatment and protein extraction

HEK-293 cells stably expressing recombinant human IRS1 (HEK-293.IRS1 cells) were a gift from Dr R. Roth (Stanford University, CA, U.S.A.). The cells were maintained in Dulbecco's modified Eagle's medium with 10 % (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Grand Island, NY, U.S.A.) at 37 °C in 10 % CO_2. 3T3L1 cells were maintained and differentiated into adipocytes in an 8-day programme as described previously [34]. For experiments, cells were first incubated with vehicle (DMSO) or rosiglitazone in growth medium for 8 h and then with vehicle or rosiglitazone in serum-free Dulbecco's modified Eagle's medium overnight. DMSO or PMA (1 μM final concentration) was then added to the cells for 30 min. The cells were exposed further to 100 nM insulin or vehicle for 15 min, washed with ice-cold PBS and then subjected to lysis.

In vivo experiments with genetically obese Zucker (fa/fa) rats

Lean and obese Zucker female rats were purchased from Charles River Laboratories (Wilmington, MA, U.S.A.). Rats were kept on a 12 h light/dark cycle at constant room temperature. Conventional laboratory diet and tap water were provided *ad lib*. until 4 h before the animals were killed, when food was withdrawn. At the time of experiment, the rats were approx. 11–12 weeks old. There were eight rats per treatment group. The rats were given a dose of 30 mpk (mg/kg body weight) rosiglitazone or vehicle (0.5 % methylcellulose) via oral gavage in the morning. Twenty-four hour after the first dose (24 h treatment) or after the second dose (48 h treatment) under the *ad lib*. fed condition, blood samples were collected from the portal vein for determination of the concentrations of non-esterified fatty acids in circulation as described previously [35]. The animals were then subjected to anaesthesia (intraperitoneal injection of nemtubal sodium solution at the dose of 2.5 mg/animal) and approx. 200–300 mg of abdominal fat was removed from each rat. Insulin was then infused via portal veins at the dose of 0.5 upk (unit/kg body weight). Similar amount of abdominal fat was removed again from each of the rats 60 s after insulin infusion. Tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C. The method for removing the tissue samples from intact animals before and after insulin treatment has been used in earlier papers [10,36]. The protocol for the animal studies was approved by IACUC (Institutional Animal Care and Use Committee).

Immunoprecipitation and Western-blot analysis of cell and tissue lysates

Proteins were extracted from HEK-293 cells and 3T3L1 adipocytes in ice-cold lysis buffer [20 mM Hepes, pH 7.4/1 % Triton X-100/20 mM β-glycerophosphate/150 mM sodium chloride/1 mM sodium orthovanadate/10 mM sodium fluoride/1 × concentration of a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, U.S.A.)]. Proteins were similarly extracted from tissue samples of Zucker lean and obese rats but with the aid of a Polytron homogenizer (Fisher Scientific, Pittsburgh, PA, U.S.A.). Cell or tissue lysates were cleared by centrifugation. Protein concentrations were determined using Bradford reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.). For immunoprecipitation, lysates from adipose tissues were mixed with antiserum against total IRS1 for 2–4 h and then mixed with Protein A/G–agarose beads for another 1–4 h at 4 °C with gentle shaking. The beads were washed three times with lysis buffers. Cell lysates or immunoprecipitates were resuspended in SDS loading buffer (Invitrogen, Carlsbad, CA, U.S.A.) and separated in precast 4–12 % gradient NuPAGE SDS/PAGE gels (Invitrogen). The proteins were then transferred to PVDF membrane and probed with primary antibodies. Detection was carried out using ECF (enhanced chemifluorescence) or ECL® (enhanced chemiluminescence) Western Blotting kit (Amersham Biosciences, Piscataway, NJ, U.S.A.). Western blotting with the ECF method was performed by scanning with a Storm® gel and blot imaging system (Molecular Dynamics, Piscataway, NJ, U.S.A.) according to the manufacturer's recommendation. Western blotting using the ECL® method was

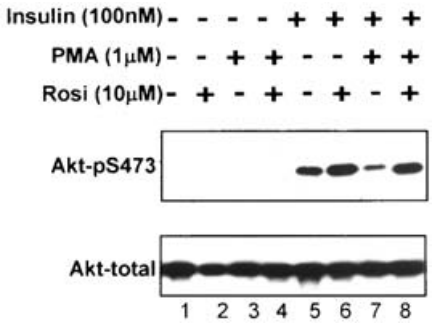

Figure 1 Reversal of PMA-induced inhibition of insulin-stimulated Akt phosphorylation by rosiglitazone in HEK-293.IRS1 cells

Cells were treated with vehicle DMSO or 10 μM rosiglitazone (Rosi) and serum-starved overnight. The cells were then treated with vehicle DMSO or 1 μM PMA for 30 min, and then exposed to vehicle acetic acid or 100 nM insulin as described in the Materials and methods section. Equivalent amounts of crude proteins were loaded on to each of the lanes. Western-blot analysis was performed using antibody against Akt-total or Akt-pS473. Similar abbreviations are used in all the Figures. Similar results were obtained in more than three independent experiments.

performed with a film according to the manufacturer's recommendation.

RESULTS

Rosiglitazone reverses PMA-induced inhibition of insulin-stimulated Akt phosphorylation in HEK-293.IRS1 cells

PMA has been shown to inhibit insulin signalling in cultured cells [15,37,38]. Akt phosphorylation (and activation) is a key event in insulin signalling and has been commonly used as a surrogate marker for assessing the levels of the activation of insulin-signalling pathway [39]. Therefore we determined whether PMA inhibits insulin-stimulated Akt phosphorylation, and if so, whether such inhibition could be reversed by rosiglitazone. As shown in Figure 1, stimulation with 100 nM insulin for 15 min increased Akt-pS473 in HEK-293 cells stably expressing recombinant human IRS1 (HEK-293.IRS1 cells) (lane 5 versus lane 1). Pretreatment with 1 μM PMA for 30 min decreased insulin-stimulated Akt phosphorylation (lane 7 versus lane 5). Pretreatment with 10 μM rosiglitazone overnight reversed the negative effects by PMA (lane 8 versus lane 7). Taken together, these results suggest that PMA inhibits insulin signalling in HEK-293.IRS1 cells and that such inhibition of PMA can be reversed by pretreatment with rosiglitazone.

Rosiglitazone reverses PMA-induced IRS1 S307 and S612 phosphorylation in HEK-293.IRS1 cells

PMA has been shown to inhibit insulin signalling by promoting inhibitory phosphorylation of IRS1 on multiple residues including S307 and S612 [15,18,40,41]. Accordingly, we determined whether the inhibitory phosphorylation of IRS1 on these sites could be reversed by rosiglitazone in the HEK-293.IRS1 cells. As shown in Figure 2, treatment with 1 μM PMA for 30 min resulted in an approx. 3-fold increase in IRS1 S307 and S612 phosphorylation. Furthermore, such phosphorylation was largely reversed by overnight pretreatment with 10 μM rosiglitazone to a level indistinguishable from that in the vehicle-treated cells. Taken together, these results showed that PMA induces IRS1 serine phosphorylation and that such inhibitory phosphorylation can

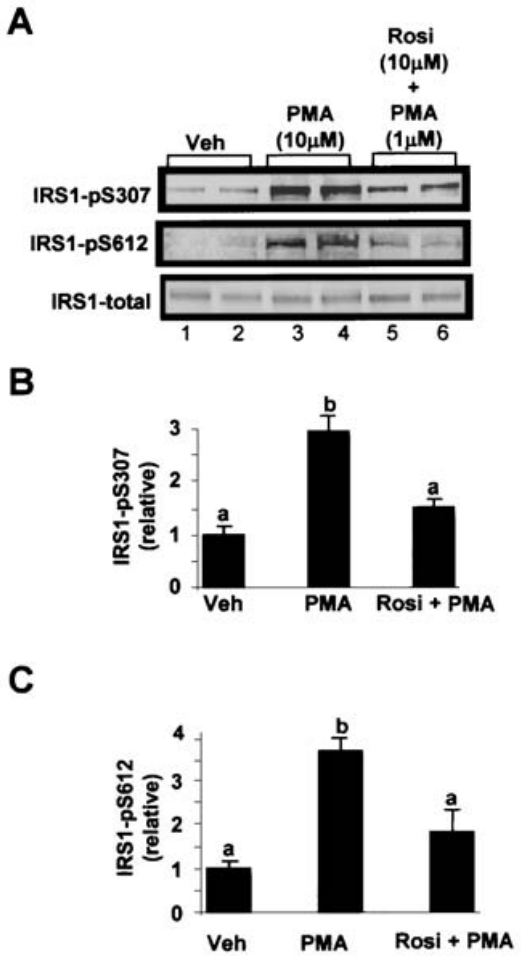

Figure 2 Reversal of PMA-induced IRS1 phosphorylation at S307 and S612 by rosiglitazone in HEK-293.IRS1 cells

Cells were maintained and treated with vehicle DMSO (Veh) or Rosi as described in Figure 1. Western-blot analysis was performed using antibody against total IRS1 (IRS1-total) or IRS1-pS307 or S612 (IRS1-pS612) (**A**). Quantification (**B**, **C**) was done using PhosphorImaging. In the bar charts, bars labelled with different letters are statistically significantly different in *t* test ($P < 0.05$). Similar labelling was used for all the Figures. Similar results were obtained in two independent experiments.

be effectively blocked by rosiglitazone treatment in the HEK-293.IRS1 cells.

Rosiglitazone reverses the effect of PMA on Akt and IRS1 phosphorylation in differentiated 3T3L1 adipocytes

HEK-293.IRS1 cells were chosen for the above study because these cells overexpress recombinant IRS1, facilitating the detection of IRS1 serine phosphorylation by Western-blot analysis. To test our hypothesis in a more physiologically relevant setting, we subsequently determined the effect of PMA and rosiglitazone on Akt and IRS1 phosphorylation in differentiated 3T3L1 adipocytes. As shown in Figure 3, stimulation with 100 nM insulin for 15 min increased Akt-pS473 in the adipocytes (lane 5 versus lane 1). Pretreatment with 1 μM PMA for 30 min decreased insulin-stimulated Akt phosphorylation (lane 7 versus lane 5). Overnight pretreatment with 10 μM rosiglitazone attenuated the negative effects of PMA on Akt phosphorylation (lane 8 versus lane 7). Taken together, these results suggest that, as in HEK-293.IRS1 cells, PMA negatively affects insulin signalling in

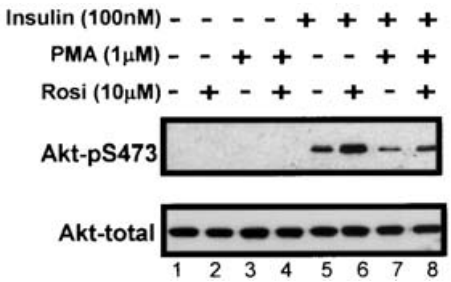

Figure 3 Reversal of PMA-induced inhibition of insulin-stimulated Akt phosphorylation by rosiglitazone in 3T3L1 adipocytes

Cells were maintained and differentiated into adipocytes by an 8-day programme as described in the Materials and methods section. As in Figures 1 and 2, the adipocytes were treated with vehicle DMSO or 10 μM Rosi and serum-starved overnight. The cells were then treated further with vehicle DMSO or 1 μM PMA for 30 min and then exposed to vehicle acetic acid or 100 nM insulin. Western-blot analysis was performed using antibody against Akt-total or Akt-pS473. Similar results were obtained in three independent experiments.

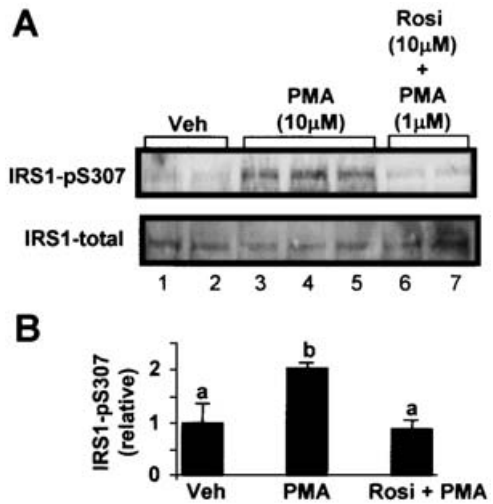

Figure 4 Reversal of PMA-induced IRS1 phosphorylation at S307 by rosiglitazone in differentiated 3T3L1 adipocytes

Cells were maintained and treated with vehicle (Veh), Rosi and PMA as described in Figure 3. Western-blot analysis was performed using antibody against IRS1-total or IRS1-pS307 (**A**). Quantification for the levels of IRS1-pS307 was done using PhosphorImaging (**B**). Similar results were obtained in three independent experiments.

3T3L1 adipocytes and such inhibition can be reversed by rosiglitazone.

As shown in Figures 4(A) and 4(B), PMA treatment resulted in approx. 2-fold increase in IRS1 S307 phosphorylation in 3T3L1 adipocytes. Furthermore, overnight pretreatment with rosiglitazone largely blocked PMA-induced IRS1 S307 phosphorylation. Taken together, these results suggest that PMA induces IRS1 S307 phosphorylation and that such inhibitory phosphorylation can be effectively blocked by rosiglitazone treatment in 3T3L1 adipocytes.

Rosiglitazone acutely potentiates insulin signalling and decreases IRS1 S307 phosphorylation in the tissues in Zucker obese rats *in vivo*

We next determined whether insulin signalling (i.e. insulin-stimulated Akt phosphorylation) is impaired and IRS1 serine

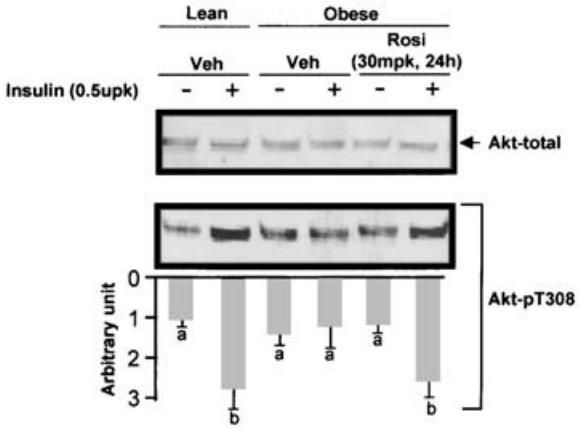

Figure 5 Acute potentiation of insulin-stimulated Akt phosphorylation in the adipose tissues of Zucker obese rats by rosiglitazone

As described in the Materials and methods section, Zucker obese rats were given one single oral dose of rosiglitazone at 30 mpk. As control, Zucker obese rats as well as lean littermates were given a dose of vehicle (Veh). Twenty-four hours post dosing and after anaesthetization, tissues from the animals were sampled immediately before and after insulin (0.5 upk) infusion. Adipose tissues were collected from the animals immediately before and after insulin infusion. Eight rats were used per treatment. For the image shown, crude lysates from the eight individual rats from a given treatment were mixed and then loaded on to a single lane in the SDS/PAGE gel. Equivalent amounts of proteins from each of the treatments were loaded on to each lane. Results are shown for Western-blot analysis on adipose tissues using antibody against Akt-total or Akt-pT308. The *t* test was based on another analysis using eight animals per treatment. Similar results were obtained in two independent experiments.

phosphorylation is increased in the adipose tissues of Zucker obese rats relative to their lean littermates. We investigated further whether rosiglitazone can improve insulin signalling and decrease IRS1 serine phosphorylation in adipose tissues in a concerted fashion. As described in the Materials and methods section, Zucker obese rats were given vehicle or rosiglitazone at 30 mpk for 24 or 48 h. After the treatment and anaesthesia, adipose tissues from the animals were sampled immediately before and after insulin infusion. As shown in Figure 5, infusion of insulin at 0.5 upk via portal vein increased Akt phosphorylation at T308 in the adipose tissue of the lean animals (lane 2 versus lane 1). In contrast, no insulin-stimulated Akt T308 phosphorylation was observed in the adipose tissues of the obese animals treated with vehicle (lane 4 versus lane 3) and this defect was corrected in the adipose tissue of the obese animals treated with rosiglitazone (lane 6 versus lane 5). The extent of increase in Akt T308 phosphorylation by insulin in the obese animals treated with rosiglitazone is similar to that in the lean animals (lanes 5 and 6 versus lanes 1 and 2). Taken together, these results suggest that insulin signalling is blunted in the adipose tissues of Zucker obese rats and can be acutely normalized by treatment with rosiglitazone.

As shown in Figure 6, Western-blot analysis showed that, on insulin stimulation, there was increased IRS1 S307 phosphorylation and decreased IRS1 tyrosine phosphorylation (lane 2 versus lane 1) in the adipose tissue of the Zucker obese rats. Furthermore, IRS1 S307 and tyrosine phosphorylation was normalized by the 24 h treatment with rosiglitazone (lane 3 versus lane 2). Taken together, these results indicate that, in adipose tissues, increased IRS1 S307 phosphorylation is correlated with insulin resistance and that the reversal of IRS1 S307 phosphorylation is correlated with insulin sensitization by rosiglitazone in Zucker obese rats *in vivo*.

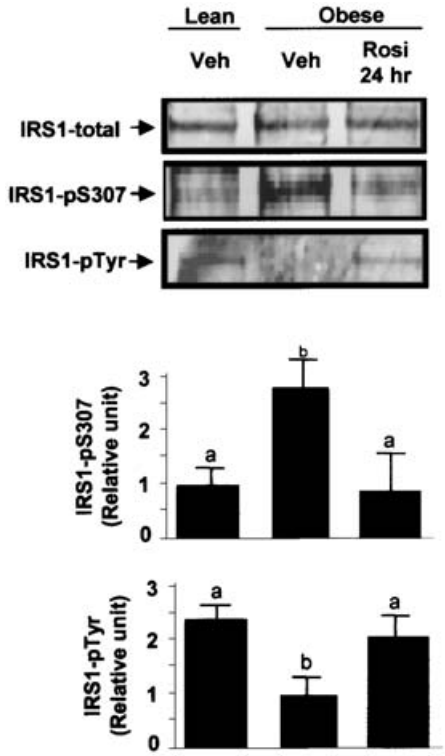

Figure 6 Reduction of IRS1 serine phosphorylation and potentiation of IRS1 tyrosine phosphorylation in the adipose tissues of Zucker obese rats by rosiglitazone

For the image shown, crude adipose lysates from eight individual rats from a given treatment after insulin infusion were mixed (1 mg of total crude proteins). The mixtures were subjected to immunoprecipitation using antisera against IRS1-total. After SDS/PAGE, Western-blot analysis was performed using antisera against IRS1-total, IRS1-pS307 or tyrosine-phosphorylated proteins. Results of the Western-blot analysis are shown. Levels of IRS1-pS307 and IRS1-pTyr (IRS1 phosphorylated tyrosine) were quantified using PhosphorImaging. The *t* test was based on another analysis using eight animals per treatment. Owing to the large number of samples (eight for each group, 24 in total), multiple gels were used for the quantitative analysis and therefore the images are not shown. Similar results were obtained in two independent experiments.

Rosiglitazone differentially inhibits p38 and JNK activation in the adipose tissues of the Zucker obese rats *in vivo*

To understand how rosiglitazone treatment decreases serine phosphorylation of IRS proteins, we determined whether rosiglitazone treatments altered the activity of potentially relevant serine/threonine kinases in the adipose tissues. As shown in Figures 7(A) and 7(B), MAPK p38 was hyper-phosphorylated (and therefore hyper-activated) in the adipose tissues of Zucker obese rats when compared with that in the lean control rats. Rosiglitazone treatment for 24 h decreased p38 phosphorylation in the adipose tissues of the obese rats to a level that was lower than that observed in the adipose tissues of the lean rats. As shown in Figures 7(A) and 7(C), JNK was also hyper-phosphorylated (and therefore hyper-activated) in the adipose tissues of Zucker obese rats when compared with that in the lean control rats. Whereas 48 h rosiglitazone treatment decreased JNK phosphorylation in the adipose tissues of the obese rats, 24 h rosiglitazone treatment did not. As shown in Figure 7(A), in contrast with p38 and JNK, MAPK p42/44 was not found to be hyper-phosphorylated in the adipose tissues of the obese rats. Furthermore, the results showed that rosiglitazone treatments did not affect p42/44 phosphorylation in the adipose tissues of obese rats.

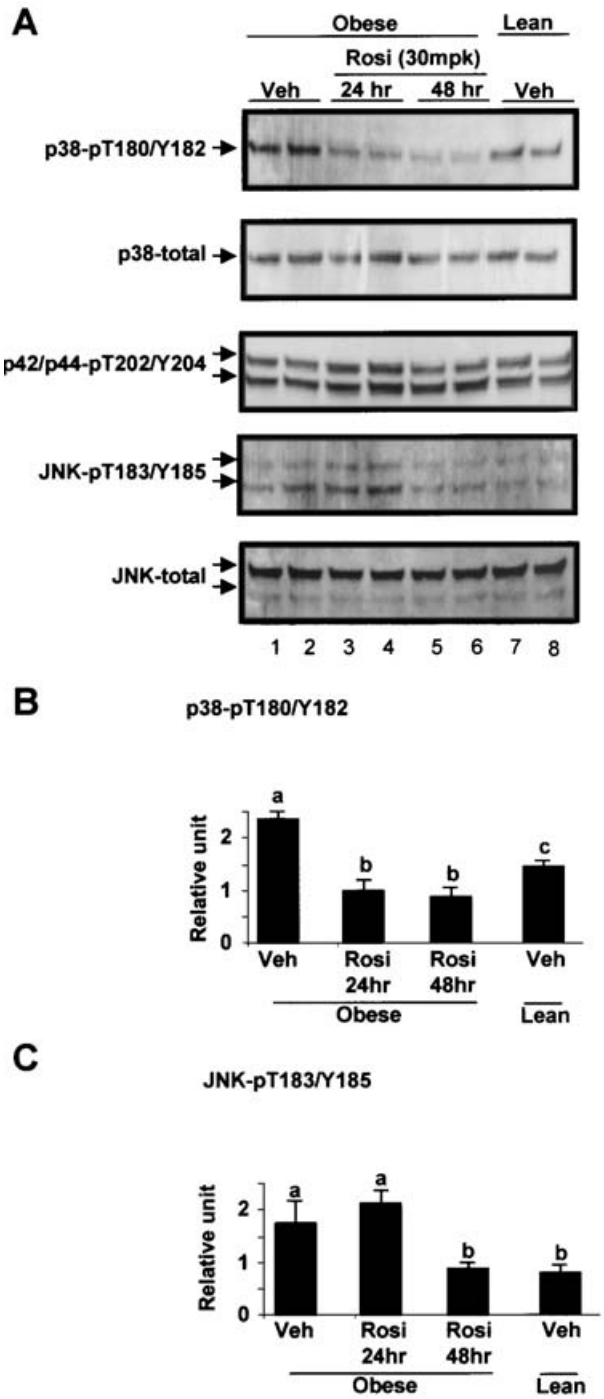

Figure 7 Inhibition of p38 and JNK phosphorylation (and activation) in the adipose tissue of Zucker obese rats by rosiglitazone

Results of Western-blot analysis using adipose tissues from animals before insulin infusion and antiserum against total p38 (p38-total), p38-pT180/Y182, p42/44-pT202/Y204, total JNK (JNK-total) and pJNK-pT183/Y185 are shown. The image includes two representative animals from each treatment. Quantification was done using PhosphorImaging. Similar results were obtained in two independent experiments.

Rosiglitazone acutely decreases circulating non-esterified fatty acid levels in the Zucker obese rats *in vivo*

It was reported that increased plasma non-esterified fatty acids led to increased intracellular levels of certain fatty acyl-CoAs

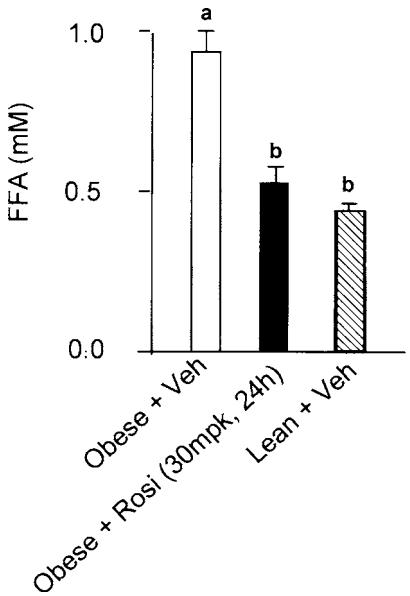

Figure 8 Acute reduction of circulating non-esterified fatty acid levels in Zucker obese rats by rosiglitazone

Non-esterified fatty acid levels of the plasma in Zucker obese rats treated with vehicle for 24 h (Veh), obese rats treated with rosiglitazone at 30 mpk for 24 h (Rosi) and control lean rats are shown. The method used for measuring non-esterified fatty acids is mentioned in the Materials and methods section. Similar results were obtained in two independent experiments.

and diacylglycerols, activation of serine/threonine kinases, increased IRS1 serine phosphorylation, decreased IRS1 tyrosine phosphorylation and insulin resistance [25]. To understand the mechanism by which rosiglitazone potentiates insulin signalling and decreases IRS S307 phosphorylation *in vivo*, we determined the effect of rosiglitazone treatment on circulating non-esterified fatty acid levels in Zucker obese rats. As shown in Figure 8, Zucker obese rats had much higher levels of circulating non-esterified fatty acids when compared with the lean control rats. Furthermore, treatment with rosiglitazone at 30 mpk for 24 h significantly decreased the level of non-esterified fatty acids in the Zucker obese rats to the level seen in the lean control rats.

DISCUSSION

It has been shown previously that PPARγ agonists, including rosiglitazone, exert their insulin-sensitizing effects at least partially by improving insulin signalling *in vitro* and *in vivo*. For instance, they promote insulin-stimulated tyrosine phosphorylation of IR and IRS1, PI3K activity associated with IRS proteins, activating phosphorylation of Akt/protein kinase B and inhibitory phosphorylation of glycogen synthase kinase 3 [10–13,42]. However, the mechanisms by which PPARγ agonists potentiate insulin signalling remain unclear. One of the possibilities is that treatment with PPARγ agonists regulates the expression of the components of the insulin-signalling cascade, i.e. IRS1 [12], the p85 subunit of the PI3K kinase [43] and c-Cbl-associated protein [44]. However, different studies examining the expression of these proteins by PPARγ agonists have yielded rather different results. Serine phosphorylation of IRS proteins is known to inhibit insulin signalling and may represent a common molecular mechanism for insulin resistance [32,45]. Accordingly, the present study examined the possibility of whether the PPARγ agonist rosiglitazone potentiates insulin signalling by reversing inhibitory serine phosphorylation of IRS proteins such as IRS1.

We demonstrated that the PPARγ agonist rosiglitazone reverses PMA-induced insulin resistance (Figure 1) and concomitantly reverses PMA-induced S307 and S612 phosphorylation of recombinant human IRS1 in HEK-293.IRS1 cells (Figure 2). These cells were chosen in the present study since the recombinant human IRS1 protein was expressed at relatively high levels, making it possible to detect readily IRS1 serine phosphorylation by Western-blot analysis. We subsequently performed similar experiments on differentiated 3T3L1 adipocytes. The results showed that, in both HEK-293.IRS1 cells and differentiated 3T3L1 adipocytes, PMA inhibits insulin-stimulated Akt phosphorylation and promotes IRS1 S307 and S612 phosphorylation and that such effects of PMA can be reversed by rosiglitazone treatment (Figures 3 and 4). Together, these results provide the first evidence that the ability of the PPARγ agonist to potentiate insulin signalling is correlated with their ability to reverse IRS S307 and S612 phosphorylation in cultured cells.

Subsequent Western-blot analyses in the present study also showed that, in adipose tissues, the levels of IRS S307 phosphorylation are significantly increased in the Zucker obese rats compared with the lean control rats (Figure 6). Similar results have been reported recently [31]. Taken together, these results strongly suggest that increased inhibitory serine phosphorylation of the IRS proteins is associated with and is likely to play a role in insulin resistance in multiple insulin responsive tissues in the disease state. Most interestingly, Western-blot analysis showed that, in adipose tissues, 24 and 48 h treatment with rosiglitazone acutely reversed IRS1 S307 hyper-phosphorylation in Zucker obese rats to the levels seen in the lean control rats (Figure 6). These results provide important evidence that a PPARγ agonist decreases IRS1 inhibitory serine phosphorylation in one of the key insulin responsive tissues *in vivo*.

It is known that IRS isoforms (IRS1–4) are expressed differentially and appear to play different roles in various insulin responsive tissues [32]. Owing to the lack of antibodies specific for serine-phosphorylated proteins of other IRS isoforms (particularly IRS2), we have not investigated whether increased inhibitory serine phosphorylation of the other IRS proteins also occurs under insulin-resistant states *in vivo* or the effect of rosiglitazone on such inhibitory phosphorylation. It will be of interest to study the regulation of IRS2 serine/threonine phosphorylation in liver since IRS1 serine phosphorylation was not detected in the present study.

There is evidence suggesting that inhibition of IRS1 serine hyper-phosphorylation may be physiologically relevant to PPARγ-agonist-mediated insulin sensitization *in vivo*. We reported previously that, in Zucker obese rats, treatment with PPARγ agonists promotes insulin-stimulated IRS1 tyrosine phosphorylation *in vivo* [10]. Serine phosphorylation is known to inhibit IRS tyrosine phosphorylation [17,20–23,30]. Accordingly, a decrease in IRS1 serine phosphorylation could directly contribute to increased insulin signalling (i.e. increased IRS1 tyrosine phosphorylation and Akt serine/threonine phosphorylation) by rosiglitazone in the Zucker obese rats as we observed. Such a proposal is supported further by the observation that the effect on IRS1 serine phosphorylation and insulin signalling by rosiglitazone *in vivo* occurred in a concomitant fashion, i.e. all within 24 h after the initiation of the treatment.

The detailed underlying mechanism by which PPARγ agonists reverse IRS1 serine phosphorylation remains unclear. It was reported previously that increased plasma non-esterified fatty acids lead to increased intracellular levels of certain fatty acyl-CoAs and diacylglycerols, activation of PKCθ, increased IRS1 S307 phosphorylation, and decreased IRS1 tyrosine phosphorylation, PI3K activation and Akt activation [24,25,46]. Since 24 h

treatment with rosiglitazone decreased the levels of circulating non-esterified fatty acids in the Zucker obese rats to the levels seen in the lean control rats (Figure 8), it is plausible that decreased IRS S307 phosphorylation in the same time frame could result from decreased non-esterified fatty acids and subsequent deactivation of certain serine/threonine kinases that are responsible for phosphorylating IRS1 in the insulin-resistant state.

In the present study, we have demonstrated that rosiglitazone treatment for 48 h also decreased JNK hyper-phosphorylation in the adipose tissues of the obese rats (Figure 7). It was reported that JNK is hyper-activated in obese animals [31] and that rosiglitazone treatment decreases the levels of the phosphorylated and activated JNK1 in the heart of diabetic rats [47]. JNK1 has been shown to bind to IRS1 and also to phosphorylate IRS1 at S307 directly *in vitro* [17,30]. JNK1 deficiency improves insulin sensitivity *in vivo* [31]. However, since the decrease in JNK hyper-phosphorylation in the adipose tissues was not observed after 24 h rosiglitazone treatment (Figure 7) when potentiation of insulin signalling was observed (Figure 6), these results suggest that the insulin-sensitizing effect of rosiglitazone was not primarily mediated by inhibition of JNK activity. On the other hand, rosiglitazone treatment for 24 h decreased hyper-phosphorylation of p38 MAPK in the adipose tissues of the obese rats (Figure 7). Since the decrease in p38 phosphorylation and the potentiation of insulin signalling in the adipose tissues occurred concomitantly within 24 h after the initiation of rosiglitazone treatment, the inhibition of p38 activation may play a role in decreasing IRS1 serine phosphorylation by rosiglitazone in the adipose tissue. Since p38 has not been shown to phosphorylate directly IRS proteins, further investigation is needed to understand the potential role of p38 inhibition in rosiglitazone-mediated insulin sensitization. It was reported that the mammalian target of rapamycin/p70 S6 kinase plays a potentially important negative role in insulin signalling by affecting IRS1 [48,49]. It was also reported that p70 S6 kinase was activated in amino acids but not lipid-induced insulin resistance in perfused rat hearts [50]. Whether p70 S6 kinase was hyper-activated in the tissues of Zucker obese rats and inhibited by rosiglitazone treatments was not investigated in the present study but warrants further investigation.

In summary, results from the present study suggest that hyper-phosphorylation of IRS1 on the serine residues occurs in adipose tissue in obese animals, and that treatment with rosiglitazone decreases IRS1 serine phosphorylation in cultured cells and in animal models, potentiates insulin signalling *in vitro* and in adipose tissues *in vivo*, decreases circulating non-esterified fatty acids *in vivo* and inhibits MAPK p38 activation in adipose tissues *in vivo*. Taken together, we propose that the reverse of IRS1 serine phosphorylation is probably physiologically relevant to rosiglitazone-mediated insulin sensitization *in vivo*. It remains to be seen whether similar effects can be observed with other PPARγ agonists, particularly those that are structurally distinct from rosiglitazone. Finally, the detailed mechanism(s) underlying the ability of rosiglitazone to decrease IRS1 serine phosphorylation also requires further investigation.

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Received 8 August 2003/24 September 2003; accepted 13 October 2003
Published as BJ Immediate Publication 13 October 2003, DOI 10.1042/BJ20031207