

Hypoglycemic Action of Thiazolidinediones/Peroxisome Proliferator-Activated Receptor γ by Inhibition of the c-Jun NH₂-Terminal Kinase Pathway

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Type 2 diabetes results from progressive pancreatic β -cell dysfunction caused by chronic insulin resistance. Activation of c-Jun NH₂-terminal kinase (JNK) inhibits insulin signaling in cultured cells and in vivo and thereby promotes insulin resistance. Conversely, the peroxisome proliferator-activated receptor (PPAR) γ synthetic ligands thiazolidinediones (TZDs) enhance insulin sensitivity. Here, we show that the TZDs rosiglitazone and troglitazone inhibit tumor necrosis factor- α -induced JNK activation in 3T3-L1 adipocytes. Our results indicate that PPAR γ mediates this inhibitory action because 1) it is reproduced by other chemically unrelated PPAR γ agonist ligands and blocked by PPAR γ antagonists; 2) it is enhanced by PPAR γ overexpression; and 3) it is abrogated by PPAR γ RNA interference. In addition, we show that rosiglitazone inhibits JNK activation and promotes the survival of pancreatic β -cells exposed to interleukin-1 β . In vivo, the abnormally elevated JNK activity is inhibited in peripheral tissues by rosiglitazone in two distinct murine models of obesity. Moreover, rosiglitazone fails to enhance insulin-induced glucose uptake in primary adipocytes from *ob/ob* JNK1^{-/-} mice. Accordingly, we demonstrate that the hypoglycemic action of rosiglitazone is abrogated in the diet-induced obese JNK1-deficient mice. In summary, we describe a novel mechanism based on targeting the JNK signaling pathway, which is involved in the hypoglycemic and potentially in the pancreatic β -cell protective actions of TZDs/PPAR γ . *Diabetes* 56:1865–1871, 2007

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AUC, area under curve; BADGE, bisphenol A diglycidyl ether; 2-DG, 2-deoxy-D-[³H]glucose; DMEM, Dulbecco's modified Eagle's medium; 15d-PGJ₂, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂; FFA, free fatty acid; F-L-Leu, *N*-(9-fluorenylmethylloxycarbonyl) leucine derivative, FMOC-L-Leucine; GTT, glucose tolerance test; JNK, c-Jun NH₂-terminal kinase; IRS-1, insulin receptor substrate-1; IL, interleukin; MAPK, mitogen-activated protein kinase; PPAR, peroxisome proliferator-activated receptor; RNAi, RNA interference; TNF- α , tumor necrosis factor- α ; TZD, thiazolidinedione.

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An early condition in the development of type 2 diabetes is insulin resistance, which is a defective response of liver, adipose tissue, and skeletal muscle to insulin. Afterward, β -cell failure is the major determinant of progression from insulin resistance to hyperglycemia. In addition to genetic predisposition and environmental factors, obesity is the greatest risk factor for the development of type 2 diabetes and is strongly associated with insulin resistance (1). Although the molecular mechanisms responsible for the development of insulin resistance in obesity are complex and not fully characterized, several molecules secreted by the adipose tissue, including tumor necrosis factor (TNF)- α , may contribute to this resistance in peripheral tissues (2). In particular, TNF- α inhibits the early steps of insulin signaling, namely insulin receptor autophosphorylation and subsequent tyrosine phosphorylation of the insulin receptor substrate (IRS)-1, by inducing serine phosphorylation of IRS-1 (3). The c-Jun NH₂-terminal kinase (JNK), which is strongly activated by TNF- α (4), downregulates insulin signaling by phosphorylation of IRS-1 on serine 307 (5). In fact, JNK activity is abnormally elevated in obesity, and its deficiency results in decreased adiposity and improvement of insulin sensitivity and insulin receptor signaling (6). Consistently, the administration of JNK inhibitors markedly improves insulin signaling in peripheral tissues and consequently reduces insulin resistance and ameliorates blood glucose homeostasis in genetically and diet-induced obese mice (7,8). In addition, JNK is involved in the loss of pancreatic β -cells induced by interleukin (IL)-1 β (9). In this regard, treatment of insulin-secreting cells or obese mice with JNK inhibitors prevents IL-1 β -induced apoptosis (10) and results in significant preservation of β -islets and improvement of insulin release in response to glucose stimulation (7), respectively. Finally, there is genetic evidence showing that increased JNK activity, as a result of the loss-of-function mutations of the scaffold protein JNK inhibitor protein-1, may cause type 2 diabetes (11). Taken together, these results strongly support the pharmacological targeting of the JNK pathway as a therapeutic approach for the treatment of diabetes (7).

Thiazolidinediones (TZDs), a class of drugs used clinically as insulin-sensitizing agents, reduce blood glucose, insulin, triglyceride, and free fatty acid (FFA) levels in animal models of insulin resistance and type 2 diabetes and in humans with these conditions (12). TZDs counteract TNF- α -induced insulin resistance by restoring insulin

receptor and IRS-1 tyrosine phosphorylation in cultured adipocytes, mice, and type 2 diabetic patients (13–17). TZDs also prevent progressive β -cell dysfunction (17,18). Several mechanisms have been proposed to account for this protective action of TZDs, including a lowering of pancreatic secretory demands (17), a direct positive effect on β -cells (19), or the inhibition of the production of pro-inflammatory cytokines (20).

TZDs are synthetic high-affinity ligands for peroxisome proliferator-activated receptor (PPAR) γ , a member of the nuclear receptor superfamily of ligand-regulated transcription factors (21), which has been implicated in distinct physiological and pathological processes, such as adipogenesis, insulin sensitivity, type 2 diabetes, atherosclerosis, inflammation, and cancer (20,22). PPAR γ can be activated by naturally occurring compounds, such as the long-chain fatty acid derivative 15-deoxy- Δ (12,14)-prostaglandin J₂ (15d-PGJ₂) (23,24). In addition to TZDs, the *N*-(9-fluorenylmethylxycarbonyl) leucine derivative, FMOC-L-leucine (F-L-Leu), is another chemically distinct synthetic ligand of PPAR γ that also shows insulin-sensitizing action in vivo (25). Although some effects of TZDs are PPAR γ independent, this nuclear receptor is the major functional receptor that mediates the pharmacological actions of these drugs, because their clinical potency correlates closely with their respective capacity to bind to this receptor (26,27). Despite the beneficial actions of TZDs, the molecular mechanism(s) by which these drugs and PPAR γ promote insulin sensitivity remains to be fully elucidated.

In this study, we show that TZDs/PPAR γ inhibit activation of the JNK pathway in cells and in vivo, and we provide evidence supporting this inhibitory action as a mechanism to conduct the hypoglycemic and potentially pancreatic β -cell protective actions of TZDs/PPAR γ .

RESEARCH DESIGN AND METHODS

Cell culture and treatments. For adipocyte differentiation, 2 days after confluency, 3T3-L1 preadipocytes were exposed for 48 h to Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 500 μ M isobutylmethylxanthine, 25 μ M dexamethasone, and 5 μ g/ml insulin followed by a 48-h incubation in DMEM supplemented with 10% FCS. Thereafter, cells were maintained in DMEM supplemented with 10% FCS. Fat accumulation was visualized by staining with Oil red O. Unless indicated, 5 days after differentiation, cells were incubated for 16 h with DMEM supplemented with 0.5% FCS and rosiglitazone (10 μ M), troglitazone (10 μ M), 15d-PGJ₂ (3 μ M), F-L-Leu (100 μ M), bisphenol A diglycidyl ether (BADGE) (10 μ M), or vehicle and stimulated with TNF- α (10 ng/ml) for 20 min before harvesting. INS-1E cells (28) were treated with rosiglitazone for 24 h or SP600125 (10 μ M) for 20 min and stimulated with IL-1 β (10 ng/ml). JNK activity and cell viability were determined after 20 min and 24 h, respectively. Viable and apoptotic cells were differentially labeled using the LIFE/DEAD Viability/Cytotoxicity kit (Molecular Probes). A minimum of 1,000 cells per condition was counted for each experiment.

Cell transfection and retroviral infection. 3T3-L1 preadipocytes seeded in 100-mm tissue culture plates were transiently transfected by lipofection (Invitrogen) and using 3 μ g each of the plasmids pCEFL-KZ-HA-JNK and pSV-Sport-PPAR γ 2 or pSV-Sport. After transfection, cells were treated as described above.

NXE cells were transfected with pSUPERretro (Oligoengine) and pSUPERretro-PPAR γ -RNAi (5'GAAGATGACAGACCTCAGG-3') by standard calcium phosphate precipitation method. Two days after transfection, tissue culture medium was filtered, supplemented with 4 μ g/ml polybrene, and used to infect 3T3-L1 preadipocytes. Infected cells were selected with puromycin (2 μ g/ml).

JNK immunocomplex assay. JNK and hemagglutinin-JNK were immunoprecipitated using the antibodies sc-474 (Santa Cruz Biotechnology) and 12CA5 (BabCO), respectively, and the JNK activity was determined as described previously (29).

Immunoblotting. IRS-1, JNK, PPAR γ , hemagglutinin-tag, and tyrosine phosphorylation were detected using the antibodies sc-559, sc-474, sc-7273 (Santa

Cruz Biotechnology), 12CA5 (BabCO), and 4G10 (Upstate Biotechnology), respectively. Immunoblots were performed using the enhanced chemiluminescence detection system.

RT-PCR analysis. RT-PCR analysis was performed using 1 μ g total RNA extracted from 3T3-L1 cells with Trizol reagent (Life Technologies) and the following primer pairs: 5'-AGAAGTCACACTCTGACAGG-3'/5'-CAATCGGATGTTCTTCGGA-3', 5'-ACTGCCTATGAGCACTTCAC-3'/CAATCGGATGGTTCTTCGGA-3', and 5'-ACCACAGTCCATGCCATCAC-3'/5'TCCACCACCCTGTTGCTGTA-3' for PPAR γ 1, PPAR γ 2, and glyceraldehyde-3-phosphate dehydrogenase, respectively.

Animals and in vivo studies. All experimental protocols were approved by the Animal Care Research Committee of the University of Barcelona. Male C57BL/6J obese (*ob/ob*) mice and lean littermates were obtained from Elevage Janvier (Le Genest-Saint-Isle, France). At 14 weeks of age, rosiglitazone (1 mg/kg) or vehicle (PBS) was orally gavaged once a day for 10 consecutive days. Thereafter, mice were anesthetized with isoflurane, and liver, skeletal muscle (hind quarter), and adipose tissue (epididymal fat pads) were excised and frozen in liquid nitrogen.

JNK1-deficient (JNK1^{-/-}) mice and wild-type littermates were obtained from parental JNK1 heterozygous mice backcrossed for five generations onto the C57BL/6J strain. For each genotype, 4-week-old male mice were divided randomly into three groups. One group was fed standard chow and the other two received a high-fat, high-carbohydrate diet (diets F4031 and F3282; BioServ). At 17 weeks of age, one of the high-fat, high-carbohydrate diet groups from each genotype was subjected to rosiglitazone treatment in the same conditions, as described above, while the rest were treated with vehicle alone. For the glucose tolerance test (GTT), on day 9 of treatment, mice were fasted for 6 h before oral administration of glucose (2 mg/g body wt), and glucose was determined in blood collected from the tail with an automatic glucometer (Elite; Bayer). The trapezoid rule was used to determine the area under curve (AUC) for plasma glucose concentrations from 1 to 60 min. Plasma insulin and triglycerides were determined by ELISA (Mercodia) and enzymatically (Accutrend GCT meter; Roche), respectively. Tissue samples were obtained on day 17 of treatment, as described above.

Adipocyte isolation and determination of glucose transport. Eight-week-old male *ob/ob*, *ob/ob* JNK1^{-/-}, and lean mice were treated with rosiglitazone (3 mg/kg) or vehicle once a day for 4 consecutive days. Thereafter, epididymal fat pads were dissected, minced in Krebs-Ringer solution supplemented with 2 mmol/l sodium pyruvate and 3% BSA, and digested with 1.5 mg/ml collagenase. Adipocytes were filtered, washed three times in the same buffer, and placed in plastic vials in a final volume of 400 μ l. In triplicates, cells were treated with vehicle, rosiglitazone, insulin, or rosiglitazone plus insulin for 10 min at 37°C before 2-deoxy-D-[³H]glucose (2-DG) was added at a final concentration of 0.1 mmol/l (0.4 μ Ci). After 10 min, 100 μ l of 100 μ M cytochalasin B was added, and adipocytes were separated by centrifugation in microtubes containing phthalic acid dinonyl ester (density 0.98 g/ml). Incorporation of labeled 2-DG was measured by liquid scintillation.

Statistics. Data were analyzed with an unpaired Student's test. Values are presented as means \pm SE; **P* < 0.05; ***P* < 0.01.

RESULTS

Rosiglitazone blocks TNF- α -induced JNK activation in 3T3-L1 adipocytes. To gain insight into the molecular mechanism behind the insulin-sensitizing action of TZDs, we examined the effect of rosiglitazone on TNF- α -induced JNK activity in 3T3-L1 adipocytes. Treatment of cells with rosiglitazone inhibited TNF- α -induced JNK activity to 52 \pm 11.8% (Fig. 1A). This action was consistent with the capacity of rosiglitazone to restore the insulin-induced tyrosine phosphorylation of IRS-1 regardless the presence of TNF- α (Fig. 1B).

PPAR γ mediates the inhibitory action of rosiglitazone on the JNK pathway. Because the antidiabetic actions of TZDs are driven, to a great extent, by PPAR γ (12), we performed a series of assays to assess whether this nuclear receptor mediates the action of rosiglitazone on the JNK pathway. Dose-response analyses performed in 3T3-L1 adipocytes showed that effective rosiglitazone doses to inhibit JNK activity correlated with those previously reported to bind to and activate PPAR γ (30) (Fig. 2A). Next, we tested several PPAR γ agonist ligands, in-

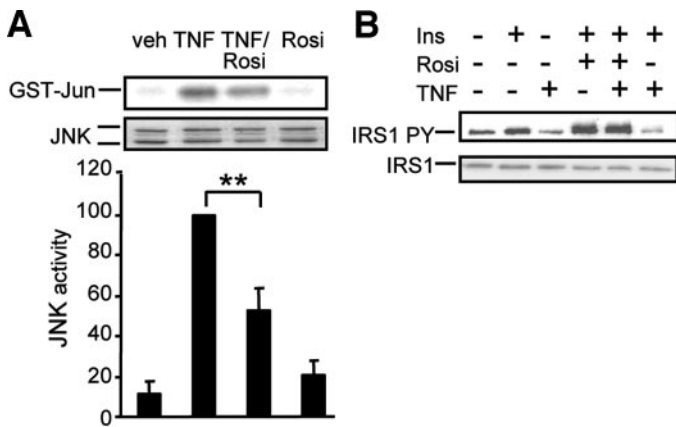


FIG. 1. Rosiglitazone inhibits TNF- α -induced activation of JNK in 3T3-L1 adipocytes. **A:** First and second panels show JNK immunocomplex and immunoblot assays from cells treated as indicated. The graph shows JNK activity referred as the percentage of the value of TNF- α -treated cells ($n = 5$). **B:** IRS-1 tyrosine phosphorylation (*top*) and total amount (*bottom*) immunoblot assay from cells treated with rosiglitazone, TNF- α , and insulin, as indicated ($n = 3$). Ins, insulin; PY, phosphotyrosine; Rosi, rosiglitazone; TNF, TNF- α ; veh, vehicle.

cluding the TZD troglitazone and other chemically distinct compounds, such as 15d-PGJ₂ and F-L-Leu, for their capacity to inhibit TNF- α -induced activation of JNK in 3T3-L1 adipocytes. JNK activity induced by this cytokine was reduced to 74 ± 10 , 69 ± 4 , and $70 \pm 11\%$ by treatment with troglitazone, 15d-PGJ₂, and F-L-Leu, respectively (Fig. 2B). Like rosiglitazone, none of these PPAR γ ligands modified basal JNK activity or the overall amount of this kinase, as assessed by immunoblot analysis (Fig. 1A; data not shown). Moreover, the downregulation of JNK activation induced by rosiglitazone in 3T3-L1 adipocytes was efficiently prevented by the PPAR γ antagonist ligand BADGE (31) (Fig. 2C). We also analyzed whether the overexpression of PPAR γ enhanced JNK inhibition by rosiglitazone in 3T3-L1 preadipocytes, which express only low levels of PPAR γ 1 (Fig. 3A). Overexpression of PPAR γ 2 in these cells improved the inhibitory action of the drug on the JNK pathway (JNK activation was decreased 32 ± 9.4 and $63 \pm 3.8\%$ in pSV and pSV-PPAR γ -transfected cells, respectively) (Fig. 3B). The PPAR γ 1 expression found in 3T3-L1 preadipocytes may account for the inhibition of JNK activity in pSV-transfected cells. Accordingly, BADGE also blocked the inhibitory action of rosiglitazone on the TNF- α -induced activity of JNK in these cells (Fig. 3C). Finally, PPAR γ expression in 3T3-L1 cells was downregulated by RNA interference (RNAi) through retroviral infection with pSUPER-PPAR γ RNAi. Effectiveness of PPAR γ RNAi was assessed by the decrease in the levels of PPAR γ mRNA (Fig. 3D) and protein (Fig. 3E) and by the inhibition of 3T3-L1 cell differentiation to adipocytes as determined by Oil red O staining (Fig. 3F). In contrast to the cells infected with the control vector, cells in which PPAR γ was downregulated by RNAi concomitantly failed to inhibit TNF- α -induced activation of JNK in response to rosiglitazone (Fig. 3G). A similar result was obtained in the infected 3T3-L1 preadipocytes (Supplemental Fig. 1, which is detailed in the online appendix [available at <http://dx.doi.org/10.2337/db06-1293>]), indicating that PPAR γ 1 also mediates the inhibitory action of rosiglitazone on the JNK pathway in these undifferentiated cells.

Rosiglitazone inhibits IL-1 β -dependent induction of JNK activity and cell death in insulin-secreting β -cell

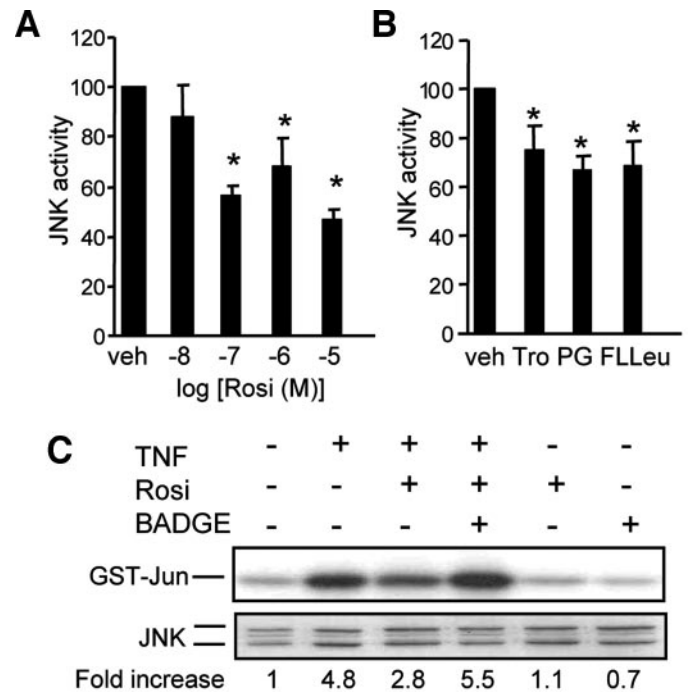


FIG. 2. Effects of PPAR γ ligands on TNF- α -induced JNK activation in 3T3-L1 adipocytes. **A** and **B:** Graphs show JNK activity as in Fig. 1A from cells treated with rosiglitazone at the concentrations indicated (**A**) or with troglitazone, 15d-PGJ₂, F-L-Leu, and stimulated with TNF- α , as indicated (**B**) ($n = 3$). **C:** JNK immunocomplex (*top*) and immunoblot (*bottom*) assays from cells treated as indicated ($n = 3$). FLLeu, F-L-Leu; PG, 15d-PGJ₂; Rosi, rosiglitazone; TNF, TNF- α ; Tro, troglitazone; veh, vehicle.

lines. In addition to the role of IL-1 β in type 1 diabetes, its glucose-induced production in β -cells contributes to β -cell glucotoxicity in the pathogenesis of type 2 diabetes (32). Because JNK plays a role in the death of pancreatic β -cells induced by IL-1 β (9), we analyzed the effect of rosiglitazone on IL-1 β -induced JNK activity and cell death in the pancreatic β -cell line INS-1E. Consistent with the PPAR γ expression in these cells, rosiglitazone efficiently inhibited JNK activity triggered by IL-1 β (Fig. 4A). Moreover, this inhibitory action correlated with the capacity of rosiglitazone to prevent IL-1 β -induced apoptosis to a similar extent as the JNK inhibitor SP600125 (Fig. 4B). Similar results were obtained in another pancreatic β -cell line, β -TC3 (Supplemental Fig. 2).

Rosiglitazone inhibits JNK activity and restores IRS-1 tyrosine phosphorylation in obese hyperglycemic *ob/ob* mice. To study the action of rosiglitazone on the JNK pathway *in vivo*, we took advantage of the exacerbated JNK activity found in peripheral tissues in the obese/hyperglycemic *ob/ob* mouse (6). Remarkably, rosiglitazone treatment significantly inhibited JNK activity in liver and adipose tissues in the *ob/ob* mice, whereas no significant effect was detected in any of these tissues in the lean littermates (Fig. 5A). In addition, extracts from liver were analyzed for IRS-1 tyrosine phosphorylation and total IRS-1 content. In agreement with a previous report (33), the amount of hepatic IRS-1 was decreased in the *ob/ob* mice. Although treatment with rosiglitazone did not restore the overall amount of IRS-1 in these animals and thus it remained lower than their lean littermates, it significantly increased IRS-1 tyrosine phosphorylation (Fig. 5B).

To analyze the effects of rosiglitazone on insulin-induced glucose uptake, primary adipocytes were isolated

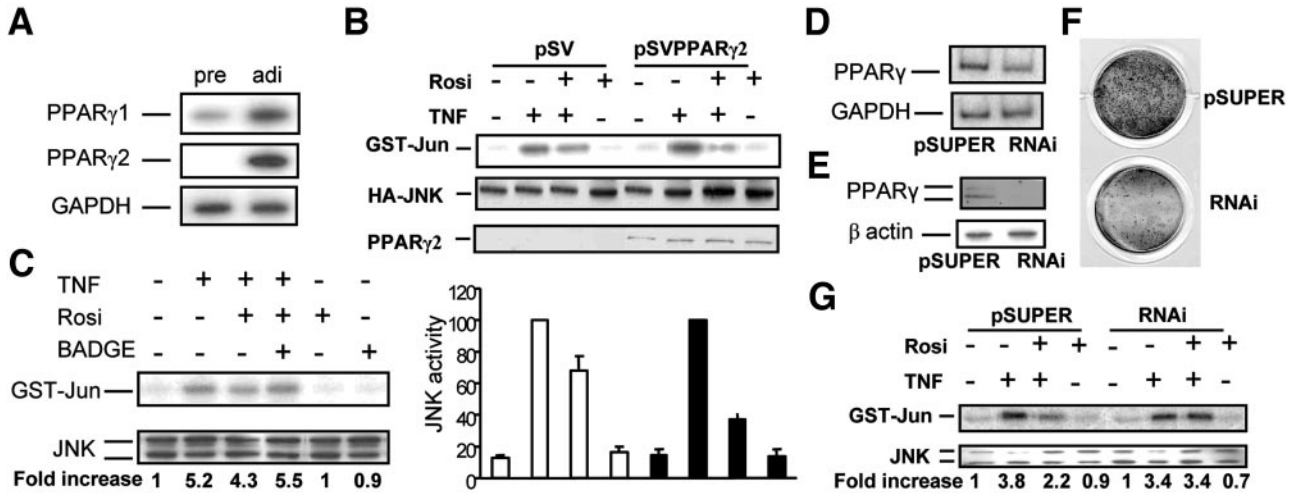


FIG. 3. Regulation of PPAR γ expression modulates rosiglitazone action on the JNK pathway in 3T3-L1 cells. *A*: RT-PCR analysis of PPAR γ 1 and -2 in 3T3-L1 preadipocytes (pre) and adipocytes (adi). *B*: HA-JNK immunocomplex (first panel), HA-JNK (second panel), and PPAR γ 2 (third panel) immunoblot assays from 3T3-L1 preadipocytes transfected with pCEFL-KZ-HA-JNK and pSV or pSV-PPAR γ 2, treated with rosiglitazone, and stimulated with TNF- α , as indicated. The graph shows the HA-JNK activity as in Fig. 1A ($n = 3$). *C*: JNK immunocomplex (top) and immunoblot (bottom) assays from 3T3-L1 preadipocytes treated as indicated. *D–G*: pSUPER (pSUPER)- or pSUPER-PPAR γ RNAi (RNAi)-infected 3T3-L1 cells were differentiated to adipocytes. Analysis of PPAR γ expression by semi-quantitative RT-PCR (*D*) and immunoblot (*E*) assays and by adipocyte differentiation (*F*). *G*: JNK immunocomplex (top) and immunoblot (bottom) assays from cells treated as indicated. Rosi, rosiglitazone; TNF, TNF- α .

from 8-week-old lean, *ob/ob*, and *ob/ob* JNK1 $^{-/-}$ mice. In contrast to lean mice, adipocytes from untreated *ob/ob* and *ob/ob* JNK1 $^{-/-}$ mice showed a reduced glucose uptake in response to insulin, which is consistent with their obesity-induced insulin resistance condition, respectively (Fig. 6). In agreement with the capacity of rosiglitazone to improve insulin sensitivity, treatment of the *ob/ob* mice with this drug clearly enhanced insulin-induced glucose uptake in primary adipocytes, whereas no effect was observed in those from lean mice. Notably, rosiglitazone failed to enhance glucose uptake in response to insulin in adipocytes from *ob/ob* JNK1 $^{-/-}$ mice (Fig. 6).

JNK1 inhibition mediates hypoglycemic action of rosiglitazone in vivo. Rosiglitazone treatment was also effective in lowering JNK activity in peripheral tissues in high-fat diet-induced obese mice (Fig. 7A). JNK1 $^{-/-}$ mice on a high-fat, high-carbohydrate diet are partially protected from adiposity (Fig. 7; Supplemental Fig. 3A); nonetheless, they still develop insulin resistance, although

milder than their wild-type littermates, as shown by GTT (Fig. 7B and C) and plasma insulin levels (Fig. 7D). In contrast to wild-type animals, treatment of JNK1 $^{-/-}$ mice with rosiglitazone neither improved their glucose tolerance (Fig. 7B and C) nor reduced their hyperinsulinemia (Fig. 7D). Regarding lipid metabolism, in response to a high-fat, high-carbohydrate diet, plasma FFAs did not increase in the JNK1 $^{-/-}$ in comparison with the wild-type mice (Supplemental Fig. 3B), whereas plasma triglycerides were found equally elevated in both types of mice. Remarkably, although the hypoglycemic action of rosiglitazone was abolished in the JNK1 $^{-/-}$ mice, another one of its beneficial actions, such as lowering plasma triglyceride

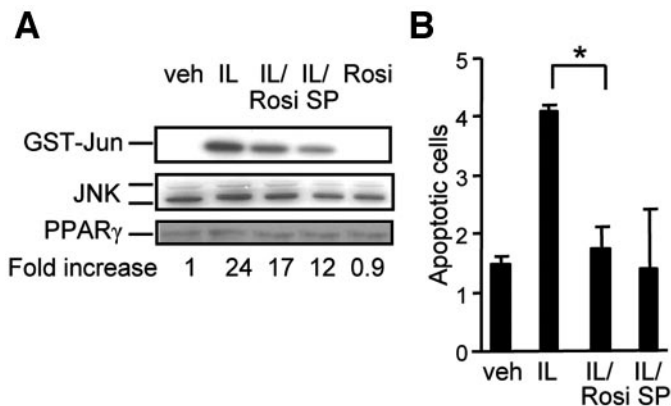


FIG. 4. Rosiglitazone inhibits IL-1 β -induced JNK activation and apoptosis in INS-1E cells. *A*: JNK immunocomplex (top) and JNK (middle) and PPAR γ (bottom) immunoblot assays from cells treated as indicated ($n = 2$). *B*: Cells treated as indicated were stained for viability. Graph shows the percentage of apoptotic cells ($n = 3$). IL, IL-1 β ; Rosi, rosiglitazone; SP, SP600125; veh, vehicle.

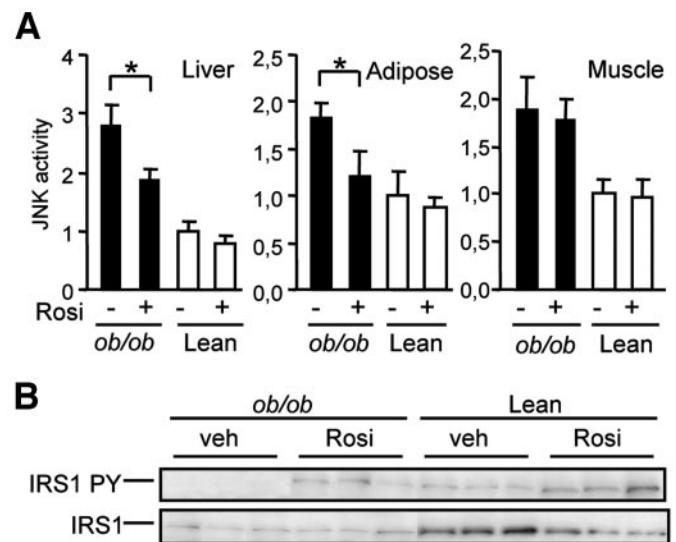


FIG. 5. Rosiglitazone inhibits JNK activity in *ob/ob* mice. *ob/ob* mice and lean littermates were treated with rosiglitazone or vehicle. *A*: Graphs show the fold increase in JNK activity in the tissues indicated relative to the untreated lean mice. In each group, $n = 5$. *B*: IRS-1 tyrosine phosphorylation (top) and total amount (bottom) immunoblot assay of liver extracts from three animals per condition. PY, phosphotyrosine; Rosi, rosiglitazone; veh, vehicle.

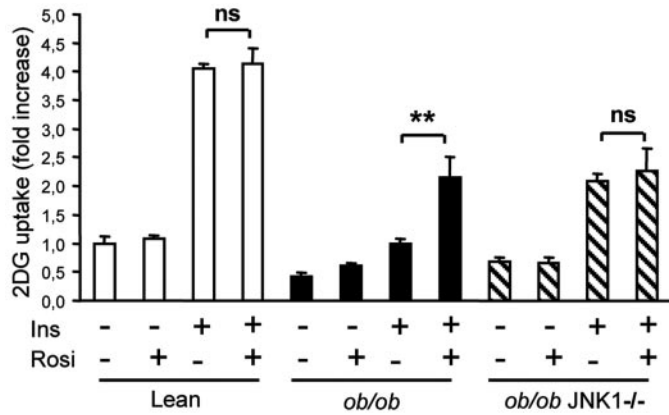


FIG. 6. Rosiglitazone fails to enhance insulin-induced glucose uptake in primary adipocytes from *ob/ob* *JNK1*^{-/-} mice. Graph shows the fold increase in 2-DG uptake in primary adipocytes isolated from lean (□), *ob/ob* (■), and *ob/ob* *JNK1*^{-/-} (▨) mice subjected to the indicated treatments relative to the untreated condition in the lean mice ($n = 3$).

level, was observed only in those mice lacking JNK1 (Fig. 7E).

DISCUSSION

Despite the pharmacological significance of TZDs, little is known about the molecular mechanism(s) behind the insulin-sensitizing action of these antidiabetic agents. These drugs ameliorate insulin resistance by acting on the early steps of the insulin signal transduction cascade, restoring insulin receptor and IRS-1 tyrosine phosphorylation levels in response to insulin in cell culture (13,14) and in vivo (this study; 15,16). This action is consistent with the inhibitory role of TZDs on the JNK pathway observed in our study given that IRS-1 is a JNK target and IRS-1 phosphorylation by JNK leads to an inhibition of insulin signaling (5). In addition, in genetically and diet-induced mouse models for obesity and diabetes, JNK inhibitors function, similarly as TZDs, as insulin-sensitizing agents, decreasing nonfasting blood glucose levels, improving glucose tolerance, decreasing fasting and nonfasting plasma insulin concentrations, and improving insulin tolerance test performance (7,8). Moreover, the failure of rosiglitazone to improve glucose uptake in response to insulin in primary adipocytes from *ob/ob* *JNK1*^{-/-} mice, and GTT and hyperinsulinemia in the high-fat diet-induced obese *JNK1*^{-/-} mice strongly supports our hypothesis that inhibition of JNK pathway is a relevant mechanism to accomplish the hypoglycemic action of TZDs. Nonetheless, it should be mentioned that not all of the beneficial actions of rosiglitazone are abolished in the *JNK1*^{-/-} mice; an effect of this drug on the high-fat diet-induced increase in plasma triglycerides is only observed in the *JNK1*-deficient mice, suggesting an inhibitory role of JNK on PPAR γ activity (see below). In this regard, it has been suggested that TZDs regulate triglyceride partitioning among various tissues independently of peripheral insulin resistance (34).

In 3T3-L1 cells, we have shown that TZDs block JNK activation by exogenously added TNF- α , indicating that the action of these drugs is exerted directly on the pathway and is not caused by a decreased production of an extracellular signaling molecule. Nonetheless, TZDs inhibit TNF- α expression in the adipose tissue of rodents, probably because of their inhibitory effects on activator protein-1 and nuclear factor- κ B activities (35,36). There-

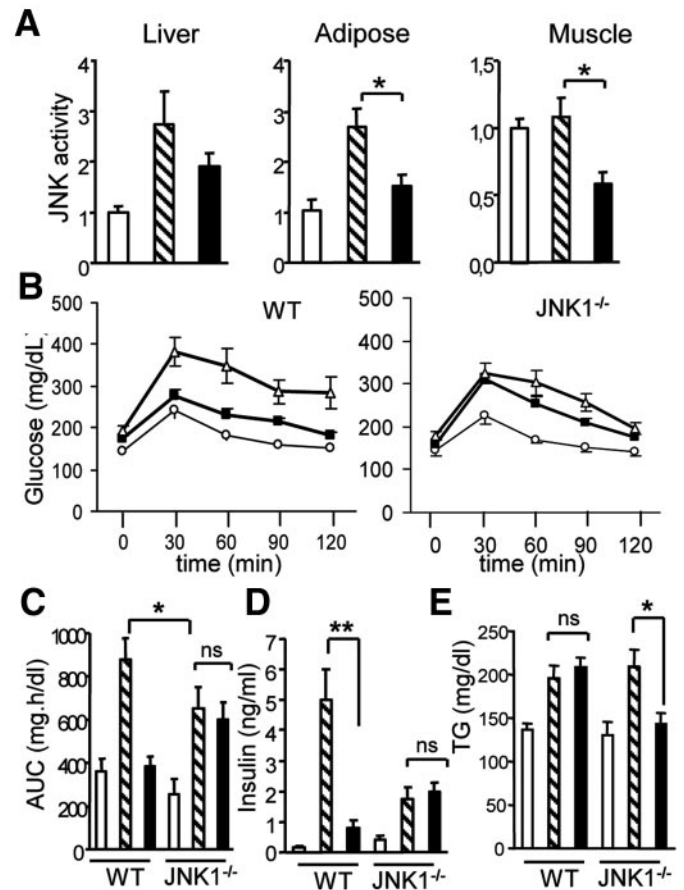


FIG. 7. Hypoglycemic action of rosiglitazone is abrogated in the high-fat diet-induced obese *JNK1*^{-/-} mice. A: Extracts from liver, adipose tissue, and skeletal muscle were obtained from control (□) and high-fat diet-induced obese mice treated with rosiglitazone (■) or vehicle (▨). Graphs show the fold increase in JNK activity relative to the untreated control mice. In each group, $n = 10$. B-D: Wild-type (WT) and *JNK1*^{-/-} mice were fed with control or high-fat, high-carbohydrate diet, and animals on high-fat, high-carbohydrate diet were treated with rosiglitazone or vehicle. In each group, $n = 10$. GTT (B), AUC for the glucose disposal curves from for GTT shown in B (C), plasma insulin (D), and triglycerides (E) from wild-type and *JNK1*^{-/-} mice on control (○ and white bars) or high-fat, high-carbohydrate diet treated with rosiglitazone (■ and black bars) or vehicle (△ and striped bars).

fore, it cannot be excluded that the inhibition of the JNK activity by TZDs in vivo is a result of two processes, a direct targeting on and a decreased production of an extracellular activator(s) of the JNK pathway. These two processes may be interdependent because, as demonstrated for other nuclear receptors, inhibition of the JNK pathway downregulates AP-1 activity (37,38), a transcriptional regulator involved in the expression of pro-inflammatory cytokine genes. In fact, rosiglitazone also inhibits lipopolysaccharide-induced activation of JNK in primary macrophages (Supplemental Fig. 4). Therefore, inhibition of the JNK pathway may also be relevant to the anti-inflammatory action of TZDs, a property that may also contribute to ameliorate insulin resistance (20). In line with this, in 3T3-L1 adipocytes and in vivo, rosiglitazone also inhibited TNF- α -induced activation and activity, respectively, of the pro-inflammatory p38 mitogen-activated protein kinase (MAPK) pathway, whereas it had no effect on extracellular-regulated protein kinase activity (Supplemental Fig. 5). Nonetheless, although rosiglitazone inhibitory action on the p38MAPK pathway may be relevant to other of its biological properties such as adipogenesis

(39), the inverse correlation between obesity and p38MAPK activity found in adipose tissue (39), liver (Supplemental Fig. 5C), and skeletal muscle (data not shown) seems to preclude the direct involvement of this protein kinase in insulin resistance and, hence, a prominent role of its inhibition by TZDs in conducting the hypoglycemic action of these drugs.

In addition to enhancing insulin sensitivity, TZDs also improve pancreatic β -cell function in type 2 diabetic patients and murine models for type 1 and type 2 diabetes (16–19). Independent studies indicate that JNK activity mediates pancreatic β -cell death in response to pro-inflammatory cytokines, such as IL-1 β (9), and that JNK inhibitors exert a protective action in these circumstances (8–10). Moreover, in the obese/hyperglycemic *db/db* mice, JNK inhibitors increase insulin gene expression (8) and delay pancreatic failure (7). In the present study, we report that the TZD rosiglitazone blocks JNK activation in response to IL-1 β in two insulin-secreting cell lines and that this action correlates with the capacity of this drug to prevent the IL-1 β -triggered death of these cells. On the basis of these preliminary observations in stable cell lines, we propose that TZDs may exert a direct protective effect on pancreatic β -cells in vivo. In addition to other indirect beneficial effects (such as lowering insulin secretory demands and pro-inflammatory cytokine levels, which eventually may also be related to the inhibitory action on the JNK pathway), this may contribute to delaying pancreatic failure, which marks the establishment of a diabetic condition.

Compelling evidence supports the notion that the insulin-sensitizing action of TZDs is mediated to a great extent by PPAR γ . In this regard, there is a strong correlation between the binding of TZDs to PPAR γ in vitro and the hypoglycemic action of these drugs in vivo (27,28). In addition, non-TZD PPAR γ ligands, such as F-L-Leu, also increase insulin sensitivity (25), and activators of the PPAR γ heterodimer partner, the retinoid X receptor, also have antidiabetic properties (40). Finally, patients who harbor a dominant-negative PPAR γ allele in heterozygosis show severe insulin resistance and diabetes (41). In the present study, we demonstrate that effective concentrations of rosiglitazone in JNK inhibition draw a parallel with those required to bind to and activate PPAR γ (30). In line with this, inhibition of JNK activation may be achieved by other TZDs, such as troglitazone, with a comparable potency to rosiglitazone, as well as, by other PPAR γ agonist ligands, such as 15d-PGJ $_2$ and F-L-Leu, and can be blocked by the PPAR γ antagonist BADGE. We also show that the action of rosiglitazone on the JNK pathway may be modulated in opposite ways by up- and downregulation of PPAR γ protein level. In this regard, the inhibitory effect of this drug on JNK activity is enhanced in 3T3-L1 preadipocytes by overexpression of PPAR γ . In contrast, PPAR γ RNAi abrogates the action of this drug on the JNK pathway in 3T3-L1 preadipocytes and adipocytes. Taken together, our data indicate that the inhibition of JNK activation by TZDs is mediated by PPAR γ .

In the light of the present study, we conclude that TZDs/PPAR γ inhibit the JNK pathway in cell cultures and in vivo. From a general point of view, this interaction should be added to the growing list of inhibitory crosstalks between the nuclear receptor superfamily and the MAPK signaling pathways. In this regard, previous studies have shown a negative regulation of PPAR γ transcriptional activity by direct JNK phosphorylation (42). Consistently,

constitutive activation of the JNK pathway by expression of a MKK7 constitutively-activated mutant leads to the inhibition of PPAR γ transcriptional activity (Supplemental Fig. 6A). The concomitant abrogation of rosiglitazone action on the JNK pathway in these conditions (Supplemental Fig. 6B) suggests that a PPAR γ transcriptional-dependent mechanism is mediating the inhibition of JNK by TZDs.

In the context of type 2 diabetes and in addition to other actions of TZDs, such as interference with the IKK/NF- κ B pathway, inhibition of the JNK pathway may be relevant for the hypoglycemic properties of TZDs/PPAR γ because exacerbated JNK activity is involved in insulin resistance and pancreatic β -cell death. In fact, after original studies showing the insulin-sensitizing and pancreatic β -cell protective activity of JNK inhibitors (7,8), current drug discovery efforts are focused on the development of molecules with such inhibitory property for the treatment of diabetes (7,43). In summary, this study contributes to a better understanding of the mechanism that underlies the pharmacological actions of TZDs and provides further support to and rationale for research on JNK and JNK inhibitors as therapeutic target and agents, respectively, for the treatment of diabetes.

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REFERENCES

1. Scheen AJ: Obesity and diabetes. In *The Management of Obesity and Related Disorders*. Kopelman PG, Ed. London, Martin Dunitz, 2001, p. 11–44
2. Flier JS: The missing link with obesity? *Nature* 409:292–293, 2001
3. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM: IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF- α - and obesity-induced insulin resistance. *Science* 271:665–668, 1996
4. Chang L, Karin M: Mammalian MAP kinase signalling cascades. *Nature* 410:37–40, 2001
5. Aguirre V, Uchida T, Yenush L, Davis R, White MF: 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, 2000
6. Hirosumi J, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS: A central role for JNK in obesity and insulin resistance. *Nature* 420:333–336, 2002
7. Bennett BL, Satoh Y, Lewis AJ: JNK: a new therapeutic target for diabetes. *Curr Opin Pharmacol* 3:420–425, 2003
8. Kaneto H, Nakatani Y, Miyatsuka T, Kawamori D, Matsuoka T, Matsuhisa M, Kajimoto Y, Ichijo H, Yamasaki Y, Hori M: Possible novel therapy for diabetes with cell-permeable JNK-inhibitor peptide. *Nat Med* 10:1128–1132, 2004
9. Ammendrup A, Maillard A, Nielsen K, Andersen NA, Serup P, Madsen OD, Mandrup-Poulsen T, Bonny C: The c-Jun amino-terminal kinase pathway is preferentially activated by interleukin-1 and controls apoptosis in differentiating pancreatic β -cells. *Diabetes* 49:1468–1476, 2000

10. Bonny C, Oberson A, Negri S, Sauser C, Schorderet DF: Cell-permeable peptide inhibitors of JNK. *Diabetes* 50:77–82, 2001
11. Waeber G, Delplanque J, Bonny C, Mooser V, Steinmann M, Widmann C, Maillard A, Miklossy J, Dina C, Hani EH, Vionnet N, Nicod P, Boutin P, Froquel P: The gene MAPK8IP1, encoding islet-brain-1, is a candidate for type 2 diabetes. *Nat Genet* 24:291–295, 2000
12. Diamant M, Heine RJ: Thiazolidinediones in type 2 diabetes mellitus. *Drugs* 63:1373–1405, 2003
13. Peraldi P, Xu M, Spiegelman BM: Thiazolidinediones block tumor necrosis factor- α -induced inhibition of insulin signaling. *J Clin Invest* 100:1863–1869, 1997
14. Iwata M, Haruta T, Usui I, Takata Y, Takano A, Uno T, Kawahara J, Ueno E, Sasaoka T, Ishibashi O, Kobayashi M: Pioglitazone ameliorates tumor necrosis factor- α -induced insulin resistance by a mechanism independent of adipogenic activity of peroxisome proliferator-activated receptor- γ . *Diabetes* 50:1083–1092, 2001
15. Miles PDG, Romeo OM, Higo K, Cohen A, Razaat K, Olefsky JM: TNF- α -induced insulin resistance in vivo and its prevention by troglitazone. *Diabetes* 46:1678–1683, 1997
16. Miyazaki Y, He H, Mandarino LJ, DeFronzo RA: Rosiglitazone improves downstream insulin receptor signaling in type 2 diabetic patients. *Diabetes* 52:1943–1950, 2003
17. Buchanan TA, Xiang AH, Peters RK, Kjos SL, Marroquin A, Goico J, Ochoa C, Tan S, Berkowitz K, Hodis HN, Azen SP: Preservation of pancreatic β -cell function and prevention of type 2 diabetes by pharmacological treatment of insulin resistance in high-risk Hispanic women. *Diabetes* 51:2796–2803, 2001
18. Leiter LA: β -Cell preservation: a potential role for thiazolidinediones to improve clinical care in type 2 diabetes. *Diabet Med* 22:963–972, 2005
19. Ogawa J, Takahashi S, Fujiwara T, Fukushige J, Hosokawa T, Izumi T, Kurakata S, Horikoshi H: Troglitazone can prevent development of type 1 diabetes induced by multiple low-dose streptozotocin in mice. *Life Sci* 65:1287–1296, 1999
20. Moller DE, Berger JP: Role of PPARs in the regulation of obesity-related insulin sensitivity and inflammation. *Int J Obes* 27:S17–S21, 2003
21. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM: The nuclear receptor superfamily: the second decade. *Cell* 83:835–839, 1995
22. Rosen ED, Spiegelman BM: PPAR- γ : a nuclear receptor of metabolism, differentiation and cell growth. *J Biol Chem* 276:37731–37734, 2001
23. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM: 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J_2 is a ligand for the adipocyte determination factor PPAR γ . *Cell* 83:803–812, 1995
24. Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM: A prostaglandin J_2 metabolite binds peroxisome proliferators-activated receptor γ and promotes adipocyte differentiation. *Cell* 83:813–819, 1995
25. Rocchi S, Picard F, Vamecq J, Gelman L, Potier N, Zeyer D, Dubuquoy L, Bac P, Champy MF, Plunket KD, Leesnitzer LM, Blanchard SG, Desreumaux P, Moras D, Renaud JP, Auwerx J: A unique PPAR γ ligand with potent insulin-sensitizing yet weak adipogenic activity. *Cell* 8:737–747, 2001
26. Willson TM, Cobb JE, Cowan DJ, Wiethe RW, Corre ID, Prakash SR, Beck KD, Moore LB, Kliewer SA, Lehmann JM: The structure-activity relationship between peroxisome proliferators-activated receptor gamma agonism and the antihyperglycemic activity of thiazolidinediones. *J Med Chem* 39:665–668, 1996
27. Berger J, Bailey P, Biswas C, Cullinan CA, Doebber TW, Hayes NS, Saperstein R, Smith RG, Leibowitz MD: Thiazolidinediones produce a conformational change in peroxisome proliferator-activated receptor gamma: binding and activation correlate with antidiabetic actions in *db/db* mice. *Endocrinology* 137:4189–4195, 1996
28. Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P: Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology* 145:667–678, 2004
29. Caelles C, Morales M: Assays to measure stress-activated mitogen-activated protein kinase activity. In *Apoptosis Methods and Protocols*. Brady HJM, Ed. Totowa, NJ, Humana Press, 2004, p. 145–156
30. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA: An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPAR γ). *J Biol Chem* 270:12953–12956, 1995
31. Wright HM, Clish CB, Mikami T, Hauser S, Yanagi K, Hiramatsu R, Serhan CN, Spiegelman BM: A synthetic antagonist for the peroxisome proliferator-activated receptor gamma inhibits adipocyte differentiation. *J Biol Chem* 275:1873–1877, 2000
32. Maedler K, Sergeev P, Ris F, Oberholzer J, Joler-Jemelka HI, Spinas GA, Kaiser N, Halban PA, Donath MY: Glucose-induced beta cell production of IL-1 β contributes to glucotoxicity in human pancreatic islets. *J Clin Invest* 110:851–860, 2002
33. Kerouz NJ, Hörsch D, Ponsm S, Kahn CR: Differential regulation of insulin receptor substrate-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (*ob/ob*) mouse. *J Clin Invest* 100:3164–3172, 1997
34. Matsui J, Terauchi Y, Kubota N, Takamoto I, Eto K, Yamashita T, Komeda K, Yamauchi T, Kamon J, Kita S, Noda M, Kadowaki T: Pioglitazone reduces islet triglyceride content and restores impaired glucose-stimulated insulin secretion in heterozygous peroxisome proliferator-activated receptor- α -deficient mice on a highfat diet. *Diabetes* 53:2844–2854, 2004
35. Ricote M, Li AC, Wilson TM, Kelly CJ, Glass CK: The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* 391:79–82, 1998
36. Jiang C, Ting AT, Seed B: PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature* 391:82–86, 1998
37. Caelles C, González-Sancho JM, Muñoz A: Nuclear hormone receptor antagonism with AP-1 by inhibition of the JNK pathway. *Genes Dev* 11:3351–3364, 1997
38. Bruna A, Nicolàs M, Muñoz A, Kyriakis JM, Caelles C: Glucocorticoid receptor-JNK interaction mediates inhibition of the JNK pathway by glucocorticoids. *EMBO J* 22:6035–6044, 2003
39. Aouadi M, Laurent K, Prot M, Le Marchand-Brustel Y, Binétruy B, Bost F: Inhibition of p38MAPK increases adipogenesis from embryonic to adult stages. *Diabetes* 55:281–289, 2006
40. Mukherjee R, Davies PJ, Crombie DL, Bischoff ED, Cesario RM, Jow L, Hamann LG, Boehm MF, Mondon CE, Nadzan AM, Paterniti JR Jr, Heyman RA: Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. *Nature* 386:407–410, 1997
41. Barroso I, Gurnell M, Crowley VE, Agostini M, Schwabe JW, Soos MA, Maslen GL, Williams TD, Lewis H, Schafer AJ, Chatterjee VK, O'Rahilly S: Dominant negative mutation in human PPAR γ associated with severe insulin resistance, diabetes mellitus and hypertension. *Nature* 402:880–883, 2001
42. Camp HS, Tafuri SR, Leff T: c-Jun N-terminal kinase phosphorylates peroxisome proliferator-activated receptor-g1 and negatively regulates its transcriptional activity. *Endocrinology* 140:392–397, 1999
43. Kaneto H: The JNK pathway as a therapeutic target for diabetes. *Expert Opin Ther Targets* 9:581–592, 2005