

# Thiazolidinediones Block Tumor Necrosis Factor- $\alpha$ -induced Inhibition of Insulin Signaling

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## Abstract

TNF- $\alpha$  has been shown to be an important mediator of insulin resistance linked to obesity. This cytokine induces insulin resistance, at least in part, through inhibition of the tyrosine kinase activity of the insulin receptor. Recently, a new class of compounds, the antidiabetic thiazolidinediones (TZDs), has been shown to improve insulin resistance in obesity and non-insulin-dependent diabetes mellitus in both rodents and man. Here we show that TZDs have powerful effects on the ability of TNF- $\alpha$  to alter the most proximal steps of insulin signaling, including tyrosine phosphorylation of the insulin receptor and its major substrate, IRS-1, and activation of PI3-kinase. Troglitazone or pioglitazone essentially eliminate the reduction in tyrosine phosphorylation of IR and IRS-1 caused by TNF- $\alpha$  in fat cells, even at relatively high doses (25 ng/ml). That this effect of TZDs operates through activation of the nuclear receptor PPAR $\gamma$ /RXR complex is shown by the fact that similar effects are observed with other PPAR $\gamma$ /RXR ligands such as 15 deoxy  $\Delta^{12,14}$ PGJ2 and LG268. The TZDs do not inhibit all TNF- $\alpha$  signaling in that the transcription factor NF- $\kappa$ B is still induced well. These data indicate that TZDs can specifically block certain actions of TNF- $\alpha$  related to insulin resistance, suggesting that this block may contribute to their antidiabetic actions. (*J. Clin. Invest.* 1997. 100:1863–1869.) Key words: insulin resistance • insulin signaling • antidiabetic drugs • NIDDM • adipocytes

## Introduction

Thiazolidinediones (TZDs)<sup>1</sup> are a new class of drugs that can be used to improve insulin resistance in type II diabetes (non-insulin-dependent diabetes mellitus, NIDDM) (1–3). One important advantage of TZDs, when compared with other drugs for NIDDM such as sulfonylureas, is that they do not induce hypoglycemia and all its related problems (4, 5). One member of the TZD class, troglitazone, is now available for NIDDM

treatment in the United States. Because these drugs decrease hyperglycemia, insulinemia, and the levels of triglycerides without directly stimulating insulin secretion, it appears that their clinical benefit is through insulin signaling improvement, which is known to be defective in NIDDM (6). Although the therapeutic effects of TZDs are becoming well-documented, their mechanism of action is only beginning to get heavy scrutiny. There is good evidence that the receptor for the antidiabetic action of the TZDs is the transcription factor PPAR- $\gamma$  (7, 8). PPAR- $\gamma$  is a nuclear hormone receptor expressed at highest levels in adipose tissue, though it is also expressed in other tissues at much lower levels. This receptor functions as an obligate heterodimer with another nuclear receptor RXR. PPAR- $\gamma$  is induced very early during adipose cell differentiation, and can act as a dominant regulator of adipocyte differentiation (9, 10). Indeed, ectopic expression of this receptor in fibroblasts induces them to differentiate into adipocytes in the presence of a PPAR- $\gamma$  ligand (9). No direct connection, however, has been made to date between PPAR- $\gamma$  activation and insulin signaling improvement.

Much evidence now indicates that TNF- $\alpha$  plays a large role in the development of insulin resistance observed linked to obesity, and perhaps to other disorders (11). Indeed, TNF- $\alpha$  mRNA is overexpressed in the adipose tissue of most animal models of obesity (12–14). TNF- $\alpha$  is also overexpressed in the fat and muscle of obese humans (15, 16). Expression of TNF- $\alpha$  mRNA correlates well with body mass index, hyperinsulinemia, and decreased lipoprotein lipase activity. That TNF- $\alpha$  plays a causal role in the insulin resistance of obese animals was demonstrated by the fact that neutralization of TNF- $\alpha$  in obese animals increases their insulin sensitivity (12). A role in humans is not yet established; one small study of patients with frank diabetes showed no effect of an anti-TNF- $\alpha$  antibody, though the effectiveness of TNF- $\alpha$  neutralization was not assessed (17). The negative effect of TNF- $\alpha$  on insulin action appears to be mediated, at least in part, through inhibition of the insulin receptor tyrosine kinase activity, since neutralization of TNF- $\alpha$  in obese animals increases the activity of the insulin receptor in fat and muscle (18). Moreover, in several cell lines, this cytokine inhibits the tyrosine kinase activity of the insulin receptor. This effect has been observed in different cell lines, including fat cells and fibroblasts (19–22). Although the precise mechanism of TNF- $\alpha$ -mediated inhibition of insulin receptor tyrosine kinase activity is unknown, several steps have been elucidated. This inhibition can be mimicked by stimulation of the p55 TNF receptor, or by treatment of cells by sphingomyelinase or ceramides, steps known to be activated by TNF- $\alpha$  (22). This inhibition leads to an increase in serine phosphorylation of insulin receptor (IR)S-1, which is converted into an inhibitor of the insulin receptor (23). In addition to these effects on insulin-signaling cascades, an effect of TNF- $\alpha$  can also be observed on the expression of Glut-4 in cultured adipocytes (24); whether this cytokine contributes to downregulation of Glut-4 in an in vivo context remains to be determined.

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Received for publication 2 June 1997 and accepted in revised form 8 August 1997.

1. Abbreviations used in this paper: IR, insulin receptor; NIDDM, non-insulin-dependent diabetes mellitus; TZD, thiazolidinediones.

J. Clin. Invest.

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0021-9738/97/10/1863/07 \$2.00

Volume 100, Number 7, October 1997, 1863–1869

http://www.jci.org

As TNF- $\alpha$  is an important mediator of insulin resistance during obesity, and as TZDs improve insulin sensitivity, we investigated the effect of TZDs on TNF- $\alpha$  signaling in cultured adipocytes. In this study, we report that TZDs can specifically and powerfully block the action of TNF- $\alpha$  to inhibit insulin signaling, suggesting one plausible mechanism for its action in improving insulin resistance.

## Methods

**Reagents.** Mouse TNF- $\alpha$  was from Genzyme Corp. (Cambridge, MA). Phosphoinositides from bovine brain were from Sigma Chemical Co. (St. Louis, MO). LG268 was a gift from Ligand Pharmaceutical (La Jolla, CA.) Troglitazone was provided by Parke Davis Warner Lambert Co. (Ann Arbor, MI), and pioglitazone was from the Upjohn Co. (Kalamazoo, MI).

**Cell culture.** 3T3-L1 cells were grown and differentiated into adipocytes as described previously (25). After maximal differentiation (typically > 90% of cells differentiated 2 wk after induction) cells were placed for 36 h in DMEM with 0.2% BSA immediately before experiments.

**Preparation of cell extracts.** After pretreatment with TZDs, cells were incubated for 6 h with TZDs and TNF- $\alpha$ , followed by a 5-min exposure to insulin ( $10^{-7}$  M). Cells were rinsed in ice-cold PBS and solubilized in stop buffer (50 mM Hepes, pH 7.2, 150 mM NaCl, 10 mM EDTA, 10 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 2 mM  $\text{Na}_3\text{VO}_4$ , 100 mM NaF, 1% Triton X-100 [vol/vol], 10  $\mu\text{g}/\text{ml}$  aprotinin, 20  $\mu\text{M}$  leupeptin, 0.18 mg/ml phenylmethylsulfonyl fluoride, and 1 mM benzamide). Equal amounts of protein were then submitted to SDS-PAGE under reducing conditions on a 7.5% polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA), and Western blot analysis was performed using the ECL Western blot kit (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

**Phosphatidylinositol 3-kinase assay.** IRS-1 was immunoprecipitated from cell extracts, and beads were washed twice with the following buffers: (a) PBS pH 7.4, 1% NP40; (b) 100 mM Tris pH 7.4, 0.5 M LiCl; and (c) 10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA. The pellets were suspended in 30  $\mu\text{l}$  of 20 mM Hepes pH 7.4, 0.4 mM EGTA, and 0.4 mM  $\text{Na}_2\text{HPO}_4$ . The kinase reaction was initiated by adding phosphatidylinositol (0.2 mg/ml), 10 mM  $\text{MgCl}_2$ , and 50 mM ( $\gamma\text{-}^{32}\text{P}$ ) ATP (10 Ci/mM). The reaction was stopped after 15 min at room temperature by adding 15  $\mu\text{l}$  of HCl 4 N, and the phosphoinositides were extracted with 130  $\mu\text{l}$  of chloroform:methanol (1:1). Phosphoinositides were analyzed by thin-layer chromatography followed by autoradiography.

**Electrophoretic mobility shift assay.** After appropriate treatment of 3T3-L1 adipocytes, cells were rinsed twice with PBS, and then scraped into 10 mM Hepes, pH 7.5, 10 mM KCl, 0.1 M EDTA, 0.1 M EGTA, 1 mM DTT, and 1 mM PMSF. After 15 min on ice, 0.6% NP40 was added. Cells were vortexed and spun for 5 min, and nuclei were resuspended in 20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, and 1 mM EGTA for 30 min at 4°C. After a 15-min spin, cell lysates were resuspended in 20 mM Hepes, pH 7.6, 1 mM EDTA, 1 mM DTT, 50 mM KCl, and 5% glycerol. The DNA binding assay was initiated by incubating 10  $\mu\text{g}$  of each cell extract with 4 mg of poly-dIdC and  $5 \times 10^{-4}$  cpm of a DNA-labeled probe containing two binding sites for NF- $\kappa\text{B}$  (ATCAGGGACTTTCCGCTGGGGACTTTCCG). After 20 min at room temperature, samples were loaded on a 4% acrylamide gel. After electrophoresis, the gel was dried and autoradiographed.

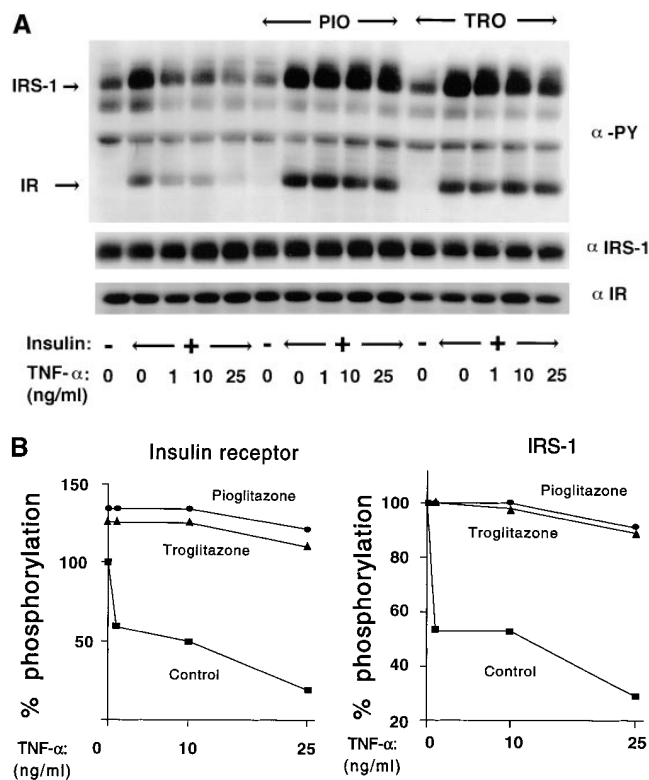
**In vitro reconstitution experiments.** Partially purified insulin receptors (26) were incubated with  $10^{-7}$  M insulin for 45 min at room temperature in Hepes, 30 mM, pH 7.2, 30 mM NaCl, 0.1% Triton X-100 (vol/vol) and were incubated with IRS-1 obtained after immunoprecipitation from cell extract, obtained as described above. Autophosphorylation of the insulin receptor was initiated by adding 15  $\mu\text{M}$

( $\gamma\text{-}^{32}\text{P}$ ) ATP, 8 mM  $\text{MnCl}_2$ , and 4 mM  $\text{MgCl}_2$  for 1 h at room temperature. The reaction was stopped by adding Laemmli buffer, and was analyzed by SDS-PAGE.

## Results

### TZDs block TNF- $\alpha$ -mediated inhibition of insulin signaling.

It has been shown that TNF- $\alpha$  inhibits insulin-induced insulin receptor and IRS-1 tyrosine phosphorylation in adipocytes and other cell types (19–22). To examine the effect of TZDs on this process, 3T3-L1 adipocytes were treated overnight with or without two different TZDs: pioglitazone or troglitazone. Cells were then treated for 6 h with various doses of TNF- $\alpha$ , stimulated with insulin for 5 min, and lysates were analyzed by Western blot using antibodies to phosphotyrosine, insulin receptor, or IRS-1 (Fig. 1 A). As expected, TNF- $\alpha$  inhibits in a dose-dependent manner (up to 80%, Fig. 1 B) tyrosine phosphorylation of insulin receptor and IRS-1 induced by in-



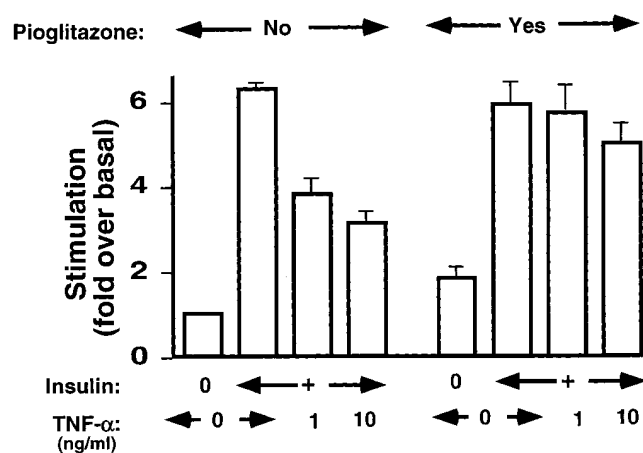
**Figure 1.** Pioglitazone and troglitazone block TNF- $\alpha$ -mediated inhibition of insulin signaling. (A) 3T3-L1 adipocytes were incubated in serum-free complete Cellgro medium for 24 h, and were then treated overnight with or without pioglitazone (5  $\mu\text{M}$ ) or troglitazone (5  $\mu\text{M}$ ). Cells were then incubated for 6 h with 1, 10, or 25 ng/ml TNF- $\alpha$ , before being stimulated with insulin ( $10^{-7}$  M) for 5 min. Lysates were analyzed by Western blot using antiphosphotyrosine ( $\alpha\text{-PY}$ ), anti-IRS-1 ( $\alpha\text{-IRS-1}$ ), or antiinsulin receptor ( $\alpha\text{-IR}$ ) antibodies. Results are representative of experiments performed at least four times. (B) The antiphosphotyrosine Western blot from Fig. 1 A was scanned using a ScanJet 4c/T (Hewlett Packard Co., Palo Alto, CA) scanner, and the intensity of the signals corresponding to the insulin receptor and IRS-1 were quantified using National Institutes of Health Image 1.60. The results are plotted as % phosphorylation (100% being the intensity of the signal of insulin receptor or IRS-1 in presence of insulin but without TNF- $\alpha$  or TZD).

sulin. As already reported by us and others, this decrease of tyrosine phosphorylation was not due to a decrease in the quantity of insulin receptor or IRS-1 (Fig. 1 A, lower two panels). When cells were pretreated overnight with 5  $\mu$ M pioglitazone or troglitazone, there was a small but consistent increase in insulin-stimulated tyrosine phosphorylation of insulin receptor (30%, Fig. 1, A and B). Most interestingly, the ability of TNF- $\alpha$  to inhibit insulin receptor and IRS-1 tyrosine phosphorylation was almost completely eliminated. This phenomenon was not linked to changes in the absolute amounts of IRS-1 or the insulin receptor.

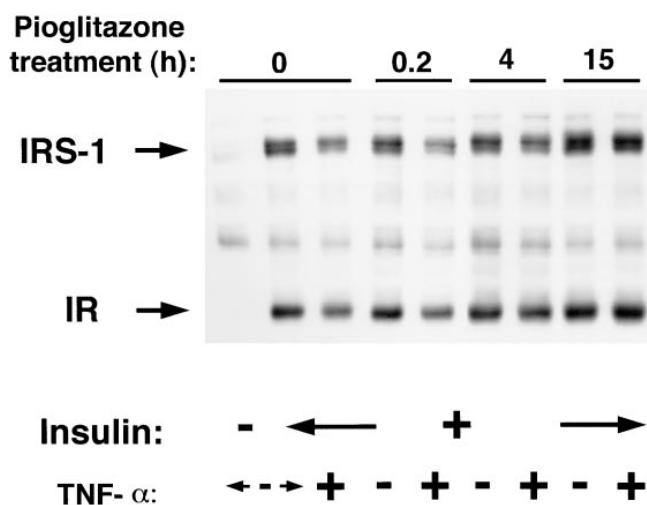
We then assessed whether this dramatic effect of the TZDs was reflected in a more downstream effect of insulin, PI3-kinase activation (Fig. 2). Activation of PI3-kinase is known to be directly implicated in insulin-dependent glucose uptake (27–29). Adipocytes were treated with or without pioglitazone overnight, then with two doses of TNF- $\alpha$ , and were then stimulated with insulin. IRS-1 was immunoprecipitated, and the associated PI-3 kinase activity was measured as described in Methods. As expected, insulin activates PI3-kinase, and this activation was decreased in a dose-dependent manner by TNF- $\alpha$  treatment. Pioglitazone induces a slight increase in this activity without insulin treatment, but did not modify significantly the insulin-induced activation of PI3-kinase. The ability of TNF- $\alpha$  to inhibit PI3-kinase, however, was almost completely eliminated in cells treated with pioglitazone. The data represent the quantification of three different experiments performed in duplicate.

These experiments indicate that exposure of cells to TZDs interferes with the ability of TNF- $\alpha$  to inhibit key proximal steps in insulin signaling.

**Time course of TZD action.** We next studied the time of exposure necessary to see the effects of the TZDs. 3T3-L1 adipocytes were treated without or with pioglitazone for 10 min, 4 h, or 15 h, and were then treated with TNF- $\alpha$  for 6 h. Cells



**Figure 2.** Pioglitazone decreases the ability of TNF- $\alpha$  to inhibit PI3-kinase. 3T3-L1 adipocytes were treated overnight with 5  $\mu$ M pioglitazone, then with 1 or 10 ng/ml of TNF- $\alpha$  before being stimulated for 5 min with insulin ( $10^{-7}$ M). PI3-kinase activity was then measured as described in Methods. Three different experiments were scanned and quantified as described in Fig. 1 B. Results are plotted as fold stimulation over basal  $\pm$  SEM (basal activity being the one observed in absence of any treatment).



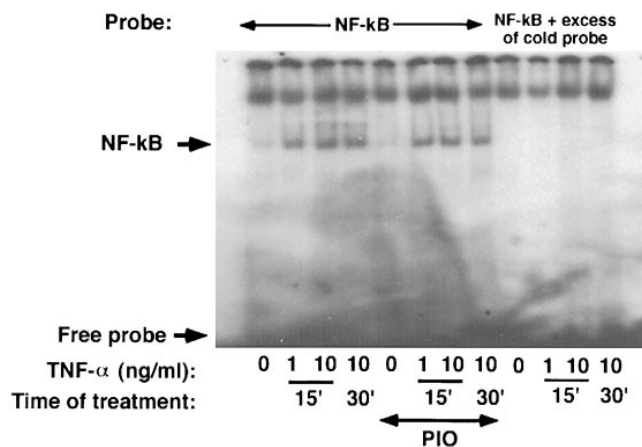
**Figure 3.** Pioglitazone needs at least four hours to protect insulin signaling from TNF- $\alpha$ . 3T3-L1 adipocytes were treated for 15 h, 4 h, or 10 min with 5  $\mu$ M pioglitazone, and were then incubated with 1 ng/ml of TNF- $\alpha$ . Cells were then stimulated with  $10^{-7}$  M insulin, lysed, and lysates were analyzed by antiphosphotyrosine Western blot. This result is representative of experiments performed three times with similar results.

were stimulated with insulin for 5 min, lysed, and cell extracts were analyzed by antiphosphotyrosine Western blot. As shown in Fig. 3, 10-min treatment with pioglitazone has no effect on TNF- $\alpha$  action, while 4-h treatment has a modest effect, reducing inhibition from 50 to 20%. By 15 h, however, TNF- $\alpha$  inhibition of these tyrosine phosphorylations are completely blocked. Thus, these actions of pioglitazone require hours of treatment, consistent with a transcriptional mechanism.

**TZD treatment does not block the activation of NF- $\kappa$ B by TNF- $\alpha$ .** TZDs may block the effect of TNF- $\alpha$  in insulin signaling by causing a global block of TNF receptor action or by affecting a part of TNF- $\alpha$  signaling that is more specifically related to inhibition of insulin action. To investigate this possibility, we tested the well-known ability of TNF- $\alpha$  to activate the transcription factor NF- $\kappa$ B in adipocytes with or without treatment with TZD. After treatments, cell nuclei were prepared and incubated with a <sup>32</sup>P-labeled DNA probe containing two binding sites for NF- $\kappa$ B, in the presence or in the absence of an excess of unlabeled probe. After a 15-min incubation the samples were submitted to an electrophoretic mobility gel shift assay (Fig. 4).

TNF- $\alpha$  induces an increase in NF- $\kappa$ B binding activity that is not modified after treatment of the cells with pioglitazone. The specificity of the NF- $\kappa$ B signals was determined by the absence of signal in presence of an excess of unlabeled probe (last four lanes). This experiment indicates that pioglitazone is not a general inhibitor of TNF- $\alpha$  action, but apparently interferes with TNF- $\alpha$ -induced insulin resistance with some specificity.

**Other ligands for PPAR $\gamma$ /RXR also block TNF- $\alpha$  effects on insulin signaling.** TZDs can serve as ligands for the transcription factor PPAR- $\gamma$ , but it is important to know whether the data presented here are the result of activation of this transcription factor. To investigate this point, we have performed similar experiments to those shown above with



**Figure 4.** Pioglitazone does not modify the ability of TNF- $\alpha$  to activate NF- $\kappa$ B. 3T3-L1 adipocytes were treated overnight with pioglitazone (5  $\mu$ M), and then for 15 or 30 min with 1 or 10 ng/ml of TNF- $\alpha$ . Nuclei were isolated, and an electrophoretic mobility shift assay using a DNA-labeled probe containing two binding sites for NF- $\kappa$ B was performed as described in Methods. The identity of the specific signal for NF- $\kappa$ B was determined by using an excess of unlabeled probe (last four lanes).

other agents of completely different chemical classes that activate the PPAR $\gamma$ /RXR complex. To do this, we used 15-deoxy $\Delta^{12,14}$ PGJ2 (15dPGJ2), a weaker but natural ligand for PPAR- $\gamma$  (8, 30), and LG268, a specific activator of RXR (31).

As shown in Fig. 5, both 15dPGJ2 and LG268 reduced inhibition of insulin receptor and IRS-1 tyrosine phosphorylation caused by TNF- $\alpha$  (inhibition decreasing from 70 to 20%). Cotreatment of cells with 15dPGJ2 and LG268 has an additive effect. These experiments strongly suggest that PPAR $\gamma$ /RXR is the complex responsible for the effect of all of these agents on insulin signaling.

*TZDs block conversion of IRS-1 into an inhibitor of the insulin receptor.* We have previously shown that IRS-1 immunoprecipitated from TNF- $\alpha$ -treated cells is associated with an inhibitory action toward the insulin receptor (23). This effect can be observed in vitro by incubating IRS-1 from TNF- $\alpha$ -treated cells with purified insulin receptors. Therefore, we studied whether or not TZDs affect this action.

3T3-L1 adipocytes were treated overnight with or without pioglitazone, and then for 6 h with TNF- $\alpha$  (10 ng/ml). Cells were lysed, and IRS-1 was immunoprecipitated and incubated in the presence of the insulin receptor. The ability of the insulin receptor to autophosphorylate was assessed as described in Methods. The insulin receptor autophosphorylates to the same extent in the control conditions (nonimmune immunoprecipitations) and in presence of IRS-1 from untreated cells (Fig. 6, A and B). As previously reported, IRS-1 from TNF- $\alpha$ -treated cells decreased the ability of insulin receptor to autophosphorylate (by 55%). The tyrosine kinase activity of the insulin receptor was not modified after incubation with IRS-1 from cells treated with pioglitazone alone. In contrast to this result, pioglitazone treatment strongly reduces the inhibitory activity associated with IRS-1 from TNF- $\alpha$ -treated cells. As expected, in each condition the pattern of phosphorylation of IRS-1 followed the phosphorylation of its enzyme, the insulin receptor.

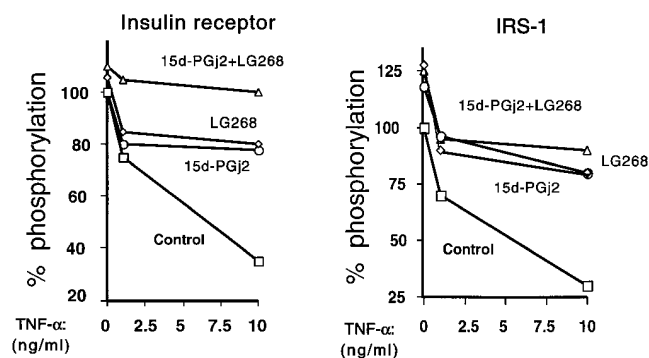
These data indicate that TZD interferes with the ability

of TNF- $\alpha$  to convert IRS-1 into an inhibitor of the insulin receptor.

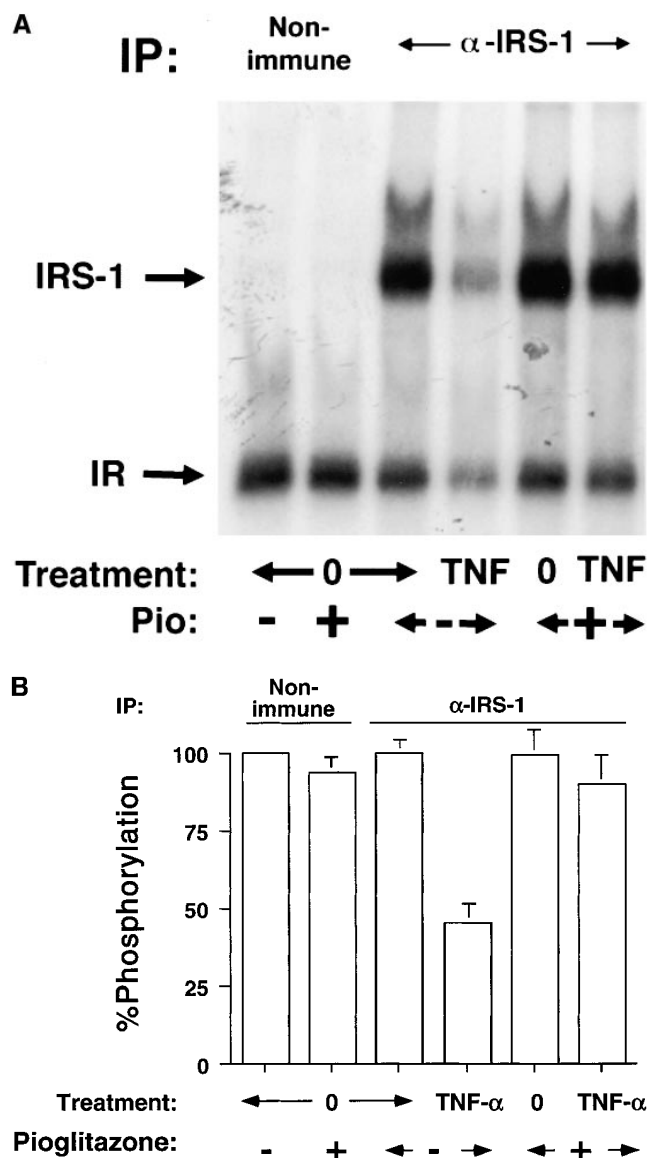
## Discussion

As the incidence of obesity has risen in the industrial world, the importance of understanding the causes and possible therapies for insulin resistance has also increased. Insulin resistance, defined as a smaller than normal response to a certain dose of insulin, contributes to several pathological problems associated with obesity, such as hyperlipidemia, arteriosclerosis, and hypertension. One of the most promising new classes of drugs is the TZDs, whose insulin-sensitizing action has been demonstrated in rodents and humans (6). A first member of this class, troglitazone, is now available in the United States for treatment of NIDDM. The mechanisms by which TZDs improve insulin resistance are largely unknown.

TZDs are ligands for the transcription factor PPAR $\gamma$ , which has a high expression in adipocytes and can function as a master regulator of adipocyte differentiation (9, 10). No direct mechanistic connection, however, has been made so far between PPAR $\gamma$  activation by TZDs and improvement of insulin sensitivity. These drugs do not seem to interfere directly with insulin secretion, suggesting that most or all of their action is on insulin signaling, which is known to be defective in obesity and NIDDM. Several studies have focused on the effect of TZDs on insulin signaling in insulin-resistant animals. TZDs increase insulin receptor tyrosine kinase activity in muscle from obese Wistar rat (32) and high fat-fed rats (33), and increase the quantity of insulin receptor and glucose transporter Glut4 in adipocytes from ob/ob mice (34). Other laboratories have focused on the effect of TZDs in cell culture. In L6 myotubes and CHO cells, TZDs potentiate insulin-induced PI-3 kinase activation without interfering with the insulin receptor tyrosine kinase activity (35). In 3T3-L1 adipocytes and L6 myotubes, TZDs increase both basal and insulin-stimulated glucose transport, correlating with enhanced expression of the glucose transporters Glut-1 and Glut-4 (36–38). These experiments were performed in cell culture where insulin signaling is normal, which may or may not give pertinent information for



**Figure 5.** Activators of PPAR- $\gamma$  or RXR protect insulin signaling from TNF- $\alpha$ . 3T3-L1 adipocytes were treated overnight with or without 15-deoxy $\Delta^{12,14}$ PGJ2 (15d-PGJ2) and LG268. Cells were treated with 1 or 10 ng/ml TNF- $\alpha$ , stimulated with insulin, and the cell lysates were analyzed by antiphosphotyrosine Western blot. A typical experiment (out of three) was scanned, and the data were plotted as described in Fig. 1 B.



**Figure 6.** Pioglitazone blocks conversion of IRS-1 into an inhibitor of the insulin receptor by TNF- $\alpha$ . 3T3-L1 adipocytes were treated overnight with pioglitazone (5  $\mu$ M), and then with 10 ng/ml of TNF- $\alpha$  for 6 h. Immunoprecipitations using IRS-1 or nonimmune antibodies were performed. After washes, beads were incubated in presence of insulin-stimulated insulin receptor, and of a phosphorylation mix (cf Methods). After 1 h, protein was analyzed by SDS-PAGE and autoradiographed. (B) Three different experiments as described in A were performed, scanned and quantified as described in Fig. 1 B, and probed as % phosphorylation  $\pm$  SEM (100% being the phosphorylation of the insulin receptor in presence of nonimmune beads).

the mode of action of TZDs during insulin resistance. Finally, TZDs interfere with the high glucose-induced inhibition of the insulin receptor by a mechanism which is still a matter of debate (21, 39). Hyperglycemia, however, is a relatively late event in the history of a typical obese/diabetic patient, making it likely that TZD have important functions in addition to preventing hyperglycemia-induced insulin resistance.

The role of TNF- $\alpha$  in the development of insulin resistance during obesity seems to be established, at least in rodents. In-

deed, TNF- $\alpha$  is overexpressed in adipocytes of obese humans and animals, and neutralization of TNF- $\alpha$  in obese animals restores a significant portion of their insulin sensitivity (12, 18). Because of this fact, we assessed whether TZDs could interfere with TNF- $\alpha$ -induced insulin resistance in adipocytes in culture. Several links have already been established between TNF- $\alpha$  and TZDs. Indeed, TZDs decrease TNF- $\alpha$  expression in adipocytes of obese animals (13). Moreover, TZDs reduce the antidifferentiation effect of TNF- $\alpha$  that is observed after long treatment of adipocytes with high concentrations of TNF- $\alpha$  (40, 41). Here, we report that two TZDs (pioglitazone and troglitazone) interfere with the ability of TNF- $\alpha$  to inhibit the insulin receptor tyrosine kinase activity. This finding is linked to an increase in IRS-1 tyrosine phosphorylation and PI3-kinase activity. The importance of these tyrosine phosphorylations are well established as crucial events in insulin signaling, as is the induction of PI3-kinase activity. Thus, these effects of TZDs would be consistent with improvement of insulin sensitivity and glucose homeostasis caused by these drugs in vivo. While this paper was under review, a new study appeared, demonstrating that TZDs can reduce the effect of infusion of TNF- $\alpha$  to cause insulin resistance in rats (42), indicating that these cell culture experiments are likely to have relevance in vivo.

PPAR- $\gamma$  is thought to be the functional receptor for the TZDs because the rank order of potency of these compounds shows a strong correlation between PPAR- $\gamma$  binding and antidiabetic effects in vivo (7, 8). In addition, no other receptor has been found. Consistent with this, we have found no effect of TZDs on TNF- $\alpha$  action in cells expressing little or no PPAR- $\gamma$  such as fibroblasts and myeloid cells (data not shown). Furthermore, the time course of the effect of TZD was consistent with complex transcriptional events: maximal after 15 h of treatment, detectable after 4 h, but undetectable after 10 min. Finally, the effect of TZD in this regard could be mimicked, albeit more weakly, by treating cells with other, less potent activators of PPAR- $\gamma$  or RXR. These results are consistent with the interesting recent work of Mukherjee et al., who showed that RXR-specific agonists are able to increase insulin sensitivity in obese mice as observed by a decrease in glycemia, lipidemia, and insulinemia (43).

At this point, the tissue targets of both TNF- $\alpha$  and TZDs are not completely clear. TNF- $\alpha$  is expressed at highest levels in adipocytes of the obese, though a recent paper suggests that muscle cells from obese patients may also produce TNF- $\alpha$  (44). Neutralization of TNF- $\alpha$  in vivo improves insulin signaling in both fat and muscle (18). It is not clear, however, whether TNF- $\alpha$  itself is binding to muscle receptors, or if molecules controlled by TNF- $\alpha$  in fat (fatty acids, for example) are the actual signaling molecule detected by muscle. A similar issue exists for the TZDs, where the receptor (PPAR- $\gamma$ ) is highest in fat, but is also expressed, albeit at very low levels, in muscle. While many more complex models can be drawn, the simplest model consistent with all these data would be for the TNF- $\alpha$  that is made by fat to act primarily on fat in an autocrine fashion, resulting in induction of cellular resistance to insulin. This model would be expected to result in higher lipolysis rates and elevated circulating free fatty acids levels. Indeed, TNF- $\alpha$  neutralization studies show that increased free fatty acids levels are a powerful result of the action of this cytokine (18). Elevated free fatty acids have been suggested to be an important agent of insulin resistance in muscle, perhaps

through the so-called Randall effect (45). Based upon the data presented here, activation of PPAR- $\gamma$  in fat by the TZD, resulting in a block in the action of TNF- $\alpha$ , may be sufficient to explain a significant portion of the action of TZDs in insulin resistance in an in vivo setting. Of course, TZDs may contribute to improve insulin sensitivity in many other ways, such as direct actions on muscle insulin sensitivity, the stimulation of more, smaller insulin-sensitive fat cells, or the regulation of leptin expression (46–48). Dissection of these physiological issues will require complex experiments, including tissue-specific knockouts of PPAR- $\gamma$ .

What is the precise mechanism by which TZDs block the effects of TNF- $\alpha$  on insulin signaling? That TNF- $\alpha$  is still able to activate the transcription factor NF- $\kappa$ B in TZD-treated cells indicates that TZDs are not general inhibitors of TNF- $\alpha$  signaling. Rather, it must affect more specifically mechanisms by which TNF- $\alpha$  signaling intersects with the insulin pathway. As shown in Fig. 6, TZDs interfere with the ability of TNF- $\alpha$  to convert IRS-1, or a complex containing IRS-1, into an inhibitor of the insulin receptor. The mechanism by which IRS-1 inhibits insulin receptor function is unknown, but correlates with the serine phosphorylation of IRS-1 (23). This serine phosphorylated IRS-1 could function by two general mechanisms: a direct steric inhibition of insulin receptor activity by IRS-1, or through an inhibitor of insulin receptor that associates with IRS-1 upon TNF- $\alpha$  treatment of cells. Therefore, TZDs could act through two obvious mechanisms: inhibition of the serine phosphorylation of IRS-1 (by activation of a phosphatase or inhibition of the kinase that phosphorylates IRS-1) or through a decrease of inhibitory activity associated with IRS-1. These questions will be more easily answered when the precise mechanism by which TNF- $\alpha$  inhibits the insulin receptor tyrosine kinase activity is known.

## Acknowledgments

We thank Dr. B. Cheatham for the gift of antibodies.

P. Peraldi is supported by a fellowship from the Juvenile Diabetes Foundation International. M. Xu is supported by a fellowship from the N.R.S.A. This work was supported by a grant from the National Institutes of Health (DK 42539).

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