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# Down-regulation by Troglitazone of Hepatic Tumor Necrosis Factor-α and Interleukin-6 mRNA Expression in a Murine Model of Non-insulin-dependent Diabetes*

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**ABSTRACT.** Troglitazone, a novel thiazolidinedione drug used to treat non-insulin-dependent diabetes mellitus, is a selective ligand for the peroxisome proliferator-activated receptor-γ (PPARγ). Recent results indicate that PPARγ activation by thiazolidinediones regulates adipose tissue- and monocyte/peritoneal macrophage-derived cytokine expression *in vitro*. We evaluated whether troglitazone may also negatively regulate cytokine expression in the liver, which harbors the majority of the body's resident macrophages but which only weakly expresses PPARγ. Lean C57BL6 mice and genetically obese KKA$^y$ mice were chronically treated with troglitazone (100 mg/kg/day for 2 weeks). At the end of treatment, hepatic expression of tumor necrosis factor (TNF)-α and interleukin (IL)-6 mRNA was quantitatively determined by kinetic polymerase chain reaction both under basal conditions and after stimulation with lipopolysaccharide (LPS). Both untreated lean and obese mice exhibited low levels of baseline TNF-α and IL-6 mRNA expression and responded with a dramatic increase in hepatic cytokine transcripts and TNF-α protein expression following a challenge with LPS. Similar to the effects on white adipose tissue, troglitazone not only down-regulated the baseline levels of hepatic TNF-α and IL-6, but also greatly attenuated the inducing effects of LPS. The extent of this inhibitory effect of troglitazone was higher in obese KKA$^y$ mice than in lean mice and was also reflected by markedly down-regulated hepatic TNF-α protein expression. These data demonstrate that chronic administration of troglitazone is associated with a greatly attenuated responsiveness towards inducers of hepatic TNF-α and IL-6 production. The possible biological consequences of these effects, however, have not yet been assessed. BIOCHEM PHARMACOL **60**;1:67–75, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** thiazolidinediones; peroxisome proliferator-activated receptor γ (PPARγ); diabetes; KKA$^y$ mice; gene regulation; cytokines

Troglitazone is a novel antidiabetic drug of the TZD‡ class which has been successfully used as an insulin sensitizer for the treatment of NIDDM. TZDs are potent and selective ligands for PPARγ. This isoform of the nuclear receptor superfamily of ligand-dependent transcription factors is predominantly expressed in the adipose tissue, where it plays a key role in adipogenesis and immune responses. In addition to its effects as a master regulator of adipocytes, PPARγ also plays a central role in the differentiation of macrophages [1].

One of the therapeutic effects of TZDs in ameliorating insulin resistance is to inhibit the pathophysiological effects exerted by TNF-α. Adipose tissue-derived TNF-α has been implicated in the pathogenesis of insulin resistance, because it inhibits tyrosine kinase activity of the insulin receptor and prevents the insulin-dependent differentiation of adipocytes. The expression of both TNF-α ligand and TNF-α receptors is elevated in white adipose tissue of genetically obese and insulin-resistant rodents and in obese humans [2–7], and peripheral levels of TNF-α protein are increased in these individuals [8]. TDZs, in turn, are able to reverse this inhibitory effect of TNF-α on insulin signaling [9] and adipocyte differentiation [10, 11], thus antagonizing the effects of TNF-α in these target tissues [6].

The molecular mechanism underlying the inhibition of TNF-α-mediated effects by TZDs has not been fully established. Nevertheless, evidence from *in vitro* studies with isolated human peripheral blood monocytes and peritoneal macrophages suggests that the principal effect of TZDs may be exerted by inhibition of TNF-α synthesis at the pretranslational level [12, 13]. These effects were mediated by the PPARγ, which in turn becomes up-regulated in acti-

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‡ *Abbreviations:* TZD, thiazolidinedione; NIDDM, non-insulin-dependent diabetes mellitus; PPARγ, peroxisome proliferator-activated receptor γ; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; LPS, lipopolysaccharide; and PCR, polymerase chain reaction.

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vated macrophages [13]. Although the liver contains the largest pool of resident macrophages in the body, studies have not yet addressed the question of whether TZDs exert a similar effect on the negative regulation of cytokines in Kupffer cells. This might be because, in contrast to the PPARα isoform, which is expressed abundantly in the liver, PPARγ is expressed at a relatively low level in liver under normal conditions [14, 15]. Therefore, the consequences of ligand binding to the PPARγ have focused on extrahepatic tissues including adipose tissue and peripheral monocytes/macrophages, and the effects of TZDs on liver function have been largely ignored.

Troglitazone has been implicated in rare adverse reactions in the liver of diabetic patients [16]. In view of the pivotal role of TNF-α and other cytokines in maintaining tissue homeostasis, we therefore sought to assess the effect of troglitazone on the regulation of TNF-α and IL-6 expression in the liver. To this extent, we used both normal mice and genetically obese ($KKA^y$) mice, with the latter featuring insulin resistance and more closely representing the human target population receiving troglitazone. In addition, expression of TNF-α and IL-6 was also determined in mice challenged with bacterial LPS in order to determine whether possible effects of troglitazone on the expression of these cytokines would also become operative after massive stimulation of TNF-α release. Our data indicate that troglitazone treatment of obese $KKA^y$ mice indeed greatly decreases the hepatic expression of TNF-α mRNA and protein and of IL-6 transcripts both under non-stimulated and LPS-stimulated conditions.

## MATERIALS AND METHODS

### *Chemicals*

All chemicals were purchased from Sigma unless specified otherwise in the text. Troglitazone was synthesized at Roche Diagnostics, with an analytical purity of 98.8%

### *Animals and Treatments*

Permission for animal studies was obtained from the local regulatory authorities, and all study protocols were in compliance with the federal guidelines. Male C57BL6J mice (specified pathogen-free; 10 weeks of age) were obtained from Biological Research Laboratories. Age-matched male $KKA^y$/TaJcl mice were obtained from Clea Japan, Inc. $KKA^y$ mice were originally produced by introducing the yellow agouti gene ($A^y$) into Japanese KK mice, resulting in a congenic lethal yellow obese mouse strain [17]. These yellow mice exhibit severe signs of obesity and NIDDM and have been widely used as a murine model both to study the pathogenesis of NIDDM [18, 19] as well as to characterize the therapeutic effects of antidiabetic agents [20–22]. The animals were housed individually in Macrolone cages with wood shavings as bedding at 20° and 50% relative humidity in a 12-hr light/dark rhythm with free access to water and KLIBA 3433 rodent pellets. They were adapted for 3 weeks to the laboratory conditions before being used for the experiments.

Lean and obese mice were treated with the vehicle consisting of 0.5% (w/v) carboxymethylcellulose, 0.5% (v/v) Tween 80, and 0.4% (v/v) benzoyl alcohol in saline or with troglitazone (100 mg/kg/day) by oral gavage for 14 days. This troglitazone dose was chosen because it reflects that dose that produces maximal hypoglycemic effects in animal models of NIDDM [23]. Twenty-four hours after the last dose of troglitazone, the mice were euthanized with $CO_2$ for TNF-α analysis. Some mice were injected with 2.5 μg/g of bacterial LPS (*Escherichia coli*, serotype 0111:B4) 24 hr after the last dose of troglitazone. Ninety minutes after LPS injection, the mice were euthanized with $CO_2$ for analysis of cytokine expression.

### *Tissue Harvesting*

Following laparotomy, the liver was quickly excised, and liver slices from all lobes were snap-frozen in liquid nitrogen and stored at −178° until used for RNA isolation and TNF-α protein measurement. White adipose tissue located around the testes and epididymides was also harvested and snap-frozen for cytokine analysis.

### *RNA Preparation*

Total hepatic RNA was isolated from small pieces of liver tissue (100 to 150 mg) with a commercial FASTARNA Kit-Green (BIO101) according to the procedures of the manufacturer. To verify the integrity and purity of the isolated RNA, its absorbance was measured at 260 and 280 nm. In addition, to ascertain RNA integrity, 10 μL of the resuspended RNA pellet was mixed with RNA loading buffer and separated by electrophoresis on a denaturing formamide/1.2% agarose gel. Ethidium bromide-stained RNA fragments were visualized by UV transillumination. The extracted RNA was stored at −80° until used for PCR.

### *Generation of cDNA Probes*

From each individual mouse, 0.3 μg/μL of total RNA was either analyzed individually or pooled for each group (N = 3) to yield 0.9 μg/μL of RNA per group. Reverse transcription into cDNA was performed in PCR tubes containing 4 μL $MgCl_2$ (25 mM), 2 μL PCR buffer II (RNA PCR Kit, Perkin Elmer), 2 μL dATP (10 mM), 2 μL dCTP (10 mM), 2 μL dGTP (10 mM), 2 μL dTTP (10 mM), 1 μL Moloney murine leukemia virus (M-MuLV) reverse transcriptase (50 U/μL), 1 μL RNase inhibitor (20 U/μL), 1 μL oligo $d(T)_{16}$ primer (50 μM), 2 μL $ddH_2O$ diethyl phosphorylcyanide, and 1 μL of template RNA. The total reaction mixture was 20 μL. The tubes were placed in a Crocodile III (Appligene-Oncor) programmed as follows: 1) 25° for 10 min, to allow for primer annealing; 2) 42° for 1 hr to perform first strand synthesis; and 3) 95° for 5 min, denaturation. The

cDNA was stored at −20° until used for quantitative PCR of the cytokines.

### *Quantitative PCR*

SYBR Green (Perkin Elmer) was used for the quantitation of PCR reactions. Upon binding to double-stranded DNA during strand elongation, SYBR Green fluorescence is greatly enhanced, making this method more sensitive than other approaches. First, the prepared cDNA (300 ng/μL) was subjected to differential polymerase chain reaction by co-amplifying for 60 cycles in the presence of 5′ and 3′ murine TNF-α and IL-6 primer pairs (MWG-Biotech). Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control to standardize the amount of cDNA added to the reaction. The following specific primer pairs were used: for TNF-α: sense primer, TCT CAT CAG TTC TAT GGC CC; antisense primer, GGG AGT AGA CAA GGT ACA AC; for IL-6: sense primer, TTC CAT CCA GTT GCC TTC TTG; antisense primer, TTG GGA GTG GTA TCC TCT GTG A; and for GAPDH: sense primer, TGT AGT GGC AAA GTG GAG ATT; antisense primer, GTG GTG CAG GAT GCA TTG CT. The sequences were obtained from Primer express software. For quantitative PCR reactions, the following components were used: 2.5 μL × 10 SYBR Green buffer, 3 μL $MgCl_2$ (25 mM), 2 μL dNTP mix containing dUTP (12.5 mM), 0.125 μL AmpliTaq Gold DNA polymerase (5 U/μL), 0.125 μL Amp Erase UNG (1 U/μL) to avoid carry over of amplified DNA, 0.1 μL sense primer (50 nM), 0.1 μL antisense primer (50 nM), 16.25 μL $ddH_2O$, and 1 μL (300 ng) of template cDNA, in a total reaction volume of 25 μL. Quantitative PCR was performed in an optical 96-well plate (PE Applied Biosystems). The wells were closed with optical cups, allowing for the measurement of fluorescence. The plates were placed in a Perkin Elmer 7700 sequence detector. The PCR reaction consisted of the following steps: 1) 50° for 2 min to prevent carry over of DNA; 2) 95° for 10 min to activate ampliTaq Gold polymerase; 3) 60 cycles each consisting of 95° for 15 sec and 60° for 1 min. In addition to the quantitative detection of the amplified DNA fragments by fluorescence, DNA was mixed with loading buffer and separated on 3% agarose gels by electrophoresis. Ethidium bromide-stained DNA fragments were visualized by UV transillumination.

### *Measurement of Hepatic TNF-α Protein*

Small liver pieces (100 mg) from vehicle- or troglitazone-treated mice were sonicated (10 sec at 14 μA) in 0.5 mL ice-cold PBS buffer. The homogenate was centrifuged at 2800 × *g* at 4° for 20 min. The crude supernatant was assayed for TNF-α bioactivity in a WEHI 164 clone 13 cytotoxicity assay, which is highly sensitive and selective for TNF-α. The cells were grown in 96-well microtiter plates in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum and 100 U/mL penicillin and streptomycin. When the cells had reached confluency, they were exposed to the liver samples or mouse recombinant TNF-α for 24 hr in a final volume of 100 μL. After the incubation period, 10 μL of a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Roche Diagnostics Cell Proliferation kit I; final concentration 0.5 mg/mL) was added to each well and incubated for another 4 hr. The precipitated formazan crystals were then solubilized overnight by the addition of 100 μL of a 10% SDS solution in 0.01 M HCl. Absorbance was measured spectrophotometrically with an ELISA plate reader at 550 nm, using 650 nm as reference wavelength. Values were calculated from a TNF-α standard curve and expressed as ng TNF-α per μg protein. Total protein content was determined with a Bio-Rad protein assay, based on the Bradford dye-binding procedure [24].

### *Statistical Analysis*

Differences between two groups were analyzed by the unpaired *t*-test (StatView). $P < 0.05$ was considered significant.

## RESULTS

With the advent of the sensitive real-time PCR method, it has become possible to reliably determine low-level expression of cytokine mRNA in the liver (Fig. 1). As expected, untreated lean mice exhibited low basal levels of hepatic TNF-α mRNA. In order to quantitate these low TNF-α transcripts, a large number of amplification cycles (>40) was needed for a fluorescent signal to become detectable during real-time PCR. In accordance with these findings, non-stimulated mice did not exhibit any detectable TNF-α protein in total liver homogenates (data not shown). Similarly, obese $KKA^y$ mice did not exhibit increased basal levels of hepatic TNF-α transcripts (90% of the expression levels determined for lean C57BL6 mice), nor was liver-associated TNF-α protein detectable under non-stimulated conditions. The findings that TNF expression in liver was not increased in obese mice are complementary to reports on increased peripheral levels of TNF-α in NIDDM [5, 7, 8], confirming that the origin of the increased amounts of circulating TNF-α in obese animals is extrahepatic.

Bacterial LPS, which is taken up by Kupffer cells, is well known to stimulate the release of a wide array of proinflammatory cytokines in the liver. Endotoxin activates a number of nuclear factors which trigger the expression of "immediate early genes," including TNF-α, followed by the production of a secondary set of cytokines, including IL-6, and other factors. Because it is widely accepted that the most relevant cytokine mediating the effects of LPS is TNF-α [25], we used LPS to stimulate hepatic TNF-α and IL-6 production. As expected, LPS treatment resulted in a steep increase in hepatic TNF-α mRNA expression. Ninety minutes postinjection, the transcript levels were >50-fold higher than those under non-stimulated conditions (Table

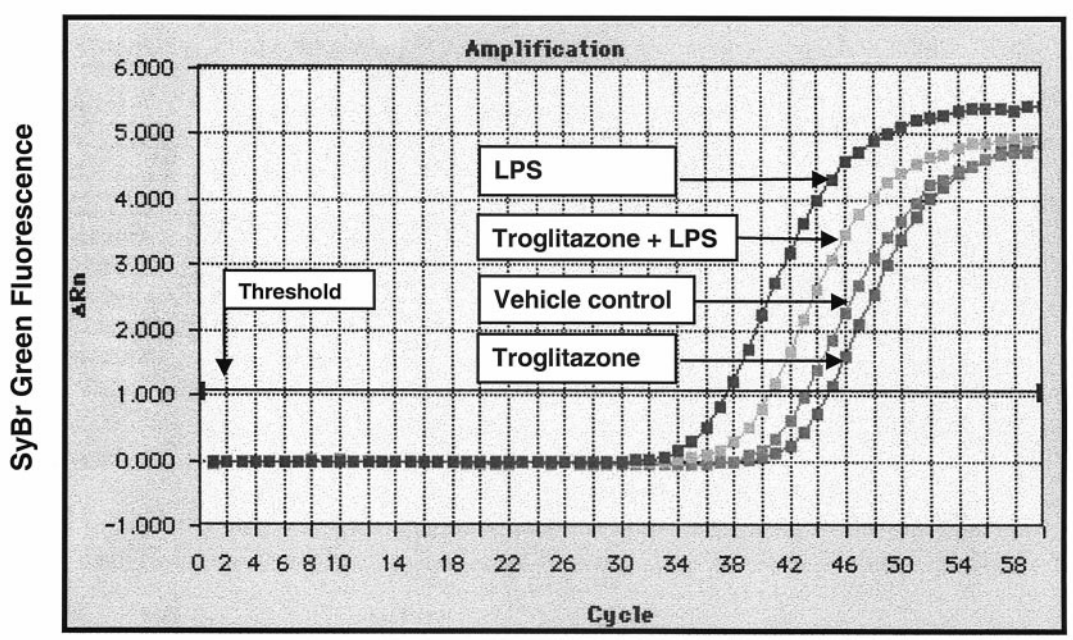


**FIG. 1. Determination of TNF-α mRNA expression levels by real-time PCR. Total RNA from vehicle- or troglitazone-treated (100 mg/kg/day, p.o., × 14 days) KKA$^y$ mice, with or without LPS treatment (2.5 μg/g), was isolated, reverse-transcribed, and amplified with TNF-α-specific primers. PCR products were incubated with SYBR Green, and the number of amplification cycles necessary for a signal to cross the threshold, set arbitrarily at 1.0, was determined. ΔRn is the difference between the detected fluorescence minus background fluorescence. The results are values of pooled cDNA from 3 individual mice.**

1). This increase in TNF-α message was paralleled by the synthesis of TNF-α protein that became detectable in liver homogenates of LPS-treated mice (Fig. 2). In the obese KKA$^y$ mice, the inducing effects of LPS on TNF-α transcription were similar in extent to those in lean control mice (Table 1). These data indicate that KKA$^y$ mice exhibit a normal response in up-regulating TNF-α and confirm previously published data obtained with ob/ob mice [7].

Next, to explore the possible modulating effects of troglitazone on hepatic cytokine expression, both lean and obese mice were treated with troglitazone, and cytokine expression was determined under both basal and stimulated conditions. Because preliminary studies with lean C57BL6 mice had revealed that a single dose of troglitazone was not sufficient to alter the transcript levels of TNF-α upon challenge with LPS, a chronic treatment study was performed. Drug treatment for 2 weeks resulted in a negative regulation of hepatic TNF-α gene expression. Although the basal levels of TNF-α transcripts were already low, the sensitive real-time PCR technique allowed for recording the reaction cycle at which the specific PCR products were first detectable (Fig. 1). Troglitazone treatment was associated with a 80% decrease in relative TNF-α mRNA levels in lean mice, paralleled by a 50% decrease in obese mice, as compared to their vehicle-treated controls. Moreover, troglitazone significantly inhibited the inducing effects of LPS on TNF-α gene expression. Figure 3 demonstrates that the hepatic expression of TNF-α mRNA was down-regulated (by 65%) by troglitazone in lean mice challenged with LPS. This effect became even more dramatic in obese mice, in which troglitazone pretreatment resulted in a massive and almost complete (93%) down-regulation of TNF-α gene expression as compared to vehicle-treated mice injected with LPS (Fig. 3). Commensurate with this inhibitory effect of troglitazone at the transcriptional level, the hepatic expression of TNF-α protein was also dramatically reduced. Troglitazone-treated KKA$^y$ mice challenged with LPS exhibited a 15-fold lower concentration of liver-associated TNF-α ligand as compared to that in vehicle-treated mice exposed to LPS (Fig. 2). This differential effect did not reach statistical significance in the corresponding lean controls due to high interindividual variation in the

**TABLE 1. Stimulation by LPS of hepatic TNF-α and IL-6 mRNA expression in lean C57BL6 mice and obese KKA$^y$ mice**

| | | Relative up-regulation (fold increase) | |
|---|---|---|---|
| Cytokine | Mice | Saline | LPS |
| TNF-α | C57BL6 | 1 | 53 |
| | KKA$^y$ | 1 | 67 |
| IL-6 | C57BL6 | 1 | 142 |
| | KKA$^y$ | 1 | 146 |

Total liver RNA from vehicle-treated (14 days) mice was isolated 90 min posttreatment with LPS (2.5 μg/g, i.p.) or saline, reverse-transcribed, and amplified with TNF-α- or IL-6-specific primers. PCR products were incubated with SYBR Green, and the number of cycles necessary to detect a fluorescence signal was determined. Relative changes in mRNA expression were calculated from these kinetic data and expressed as fold increase relative to saline-treated control mice. Data represent values of pooled cDNA from 3 mice per group.

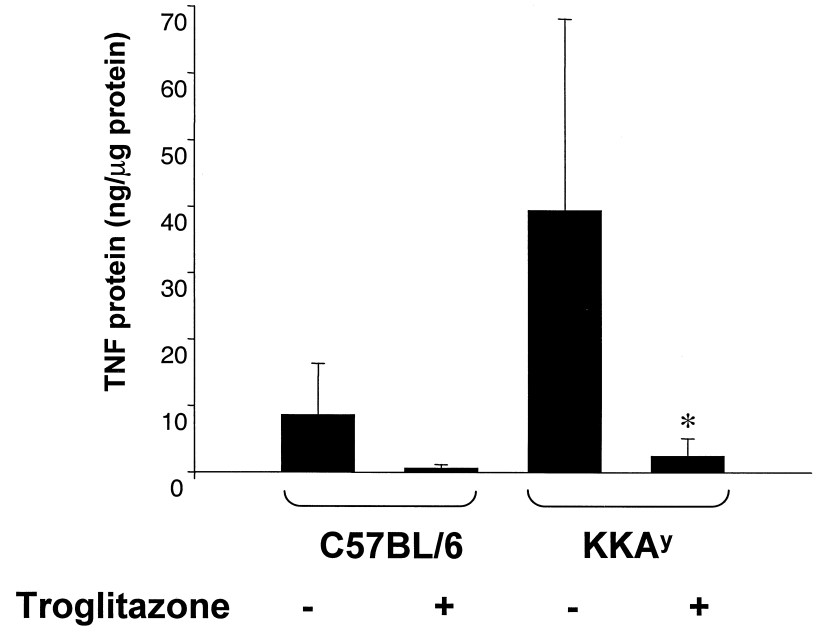


**FIG. 2. Hepatic TNF-α protein levels in lean C57BL6 mice and obese KKA$^y$ mice after LPS stimulation. TNF-α concentrations were determined by exposing WEHI cells to diluted whole-liver homogenates from vehicle- or troglitazone (100 mg/kg/day, 14 days)-treated mice prepared 90 min posttreatment with saline or LPS (2.5 μg/g). The cell numbers were determined 24 hr after addition of liver homogenate by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) activity. TNF-α concentrations were calculated from a standard curve using recombinant murine TNF-α. Data are means ± SD of 3 animals per group. *, significantly different from KKA$^y$ vehicle control ($P < 0.05$).**

response to LPS. Nevertheless, in troglitazone-treated lean mice challenged with LPS, the amount of liver-associated TNF-α ligand was greatly reduced and was close to the detection limit.

A similar qualitative effect of troglitazone pretreatment was observed for the regulation of hepatic IL-6 gene expression. As expected, vehicle control mice injected with LPS readily responded with a burst in IL-6 transcriptional activity (Table 1). However, in both obese and lean mice treated with the TZD and chal-

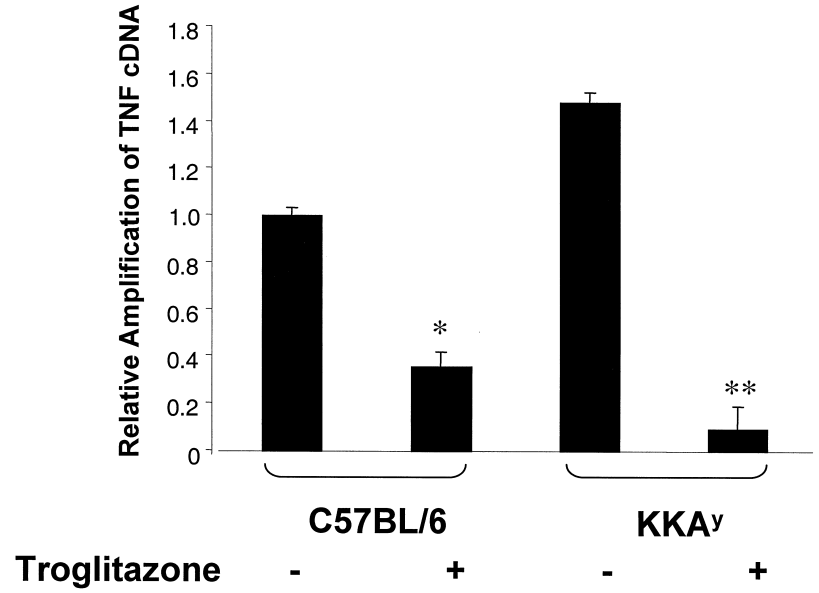


**FIG. 3. Effects of troglitazone on LPS-stimulated hepatic TNF-α expression in lean C57BL6 mice and obese KKA$^y$ mice. Total liver RNA from vehicle- or troglitazone-treated mice was isolated 90 min posttreatment with LPS (2.5 μg/g), reverse-transcribed, and amplified with TNF-α-specific primers. PCR products were incubated with SYBR Green, and the number of cycles necessary to detect a fluorescence signal was determined. Relative changes in mRNA expression were calculated from the kinetic data and expressed as increase or decrease relative to vehicle-treated lean control mice. Data are means ± SD of 3 mice per group. *, significantly different from C57BL6 vehicle control; **, significantly different from KKA$^y$ vehicle control ($P < 0.05$).**

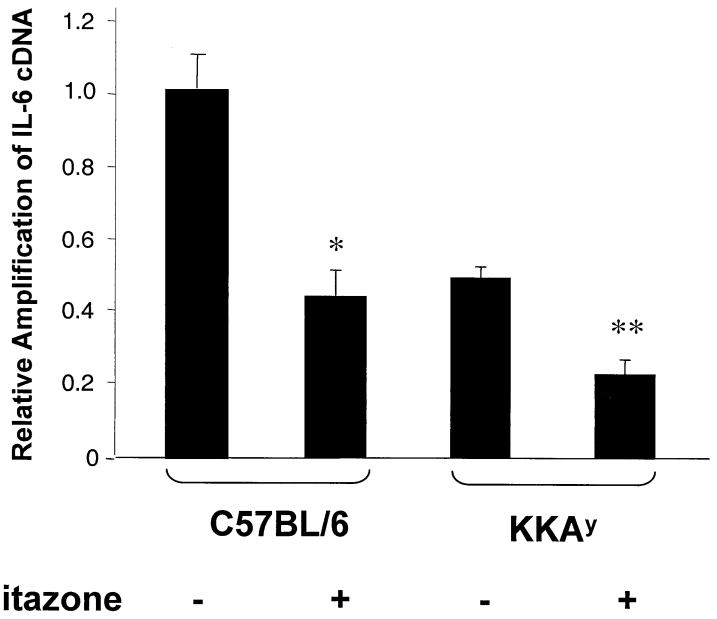


**FIG. 4. Effects of troglitazone on LPS-stimulated hepatic IL-6 expression in lean C57BL6 mice and obese KKA$^y$ mice. Total liver RNA from vehicle- or troglitazone-treated mice was isolated 90 min posttreatment with LPS (2.5 μg/g), reverse-transcribed, and amplified with IL-6-specific primers. PCR products were incubated with SYBR Green, and the number of cycles necessary to detect a fluorescence signal was determined. Relative changes in mRNA expression were calculated from these kinetic data and expressed as increase or decrease relative to vehicle-treated lean control mice. Data are means ± SD of 3 mice per group. *, significantly different from C57BL6 vehicle control; **, significantly different from KKA$^y$ vehicle control ($P < 0.05$).**

lenged with LPS, hepatic IL-6 mRNA was down-regulated by 50% (Fig. 4).

Finally, to compare the effects of troglitazone in liver with the known effects of TZDs in one of the pharmacological target tissues, changes in TNF-α and IL-6 transcript levels were determined in white adipose tissue. Following injection of LPS, vehicle-treated KKA$^y$ mice exhibited an approximately 8-fold higher expression level of TNF-α mRNA than that in similarly treated lean control mice (Fig. 5A). Troglitazone treatment almost totally reduced TNF-α transcription in these obese mice, while the corresponding levels in lean control mice were down-regulated by 60%. This inhibitory effect was paralleled by a similarly dramatic effect of the drug on IL-6 mRNA expression (Fig. 5B). The initial levels of IL-6 transcripts were similar in lean and obese mice following LPS injection alone. Troglitazone treatment completely down-regulated (by >99%) these increased IL-6 transcript levels both in lean and obese mice to almost non-detectable levels, indicating that the negative regulatory effect of troglitazone was even more intense in adipose tissue than in liver.

## DISCUSSION

This study was designed to explore the hypothesis that TZDs might be able to selectively regulate cytokine gene expression in the liver. The results demonstrate that troglitazone administration to mice indeed down-regulated hepatic TNF-α and IL-6 expression both under basal conditions and following stimulation with LPS. Furthermore, we showed that the negative regulation of both cytokines occurred at the transcriptional level. Importantly, this effect is more prominent in obese KKA$^y$ mice, a murine model of NIDDM, than in the lean control mice.

These results confirm and extend those obtained with isolated extrahepatic macrophages and monocytes *in vitro* [12, 13]. Our *in vivo* data make it unlikely that the inhibitory effects of TZDs on cytokine production may reflect a direct toxic effect due to high concentrations of the drug. In fact, troglitazone is bound to plasma proteins (>99%), and the "free" concentration is several orders of magnitude smaller than that used for eliciting an inhibitory effect on macrophages *in vitro*. Thus, our findings suggest that, similar to the drug's action in the adipose tissue, the negative regulatory effects of TZDs on hepatic proinflammatory cytokines is an adaptive process rather than a non-specific response.

The mechanism underlying this negative regulation of TNF-α and IL-6 is not known. It has been suggested that the selective effects of TZDs on cytokine gene expression are mediated by PPARγ [12, 13, 26]. In liver, however, this nuclear receptor is not abundantly expressed. Specifically, in normal murine liver, which contains two isoforms (PPARγ1 and 2) encoded by the same gene but transcribed from different promoters [27], the PPARγ1 isoform is expressed at a very low level, and the PPARγ2 form is not detectable at all [28]. One possibility is that PPARγ expression might have become induced in the KKA$^y$ mice. In fact, evidence has indicated that in obese mice fed a high-fat diet, PPARγ2 can be up-regulated, attaining 10%

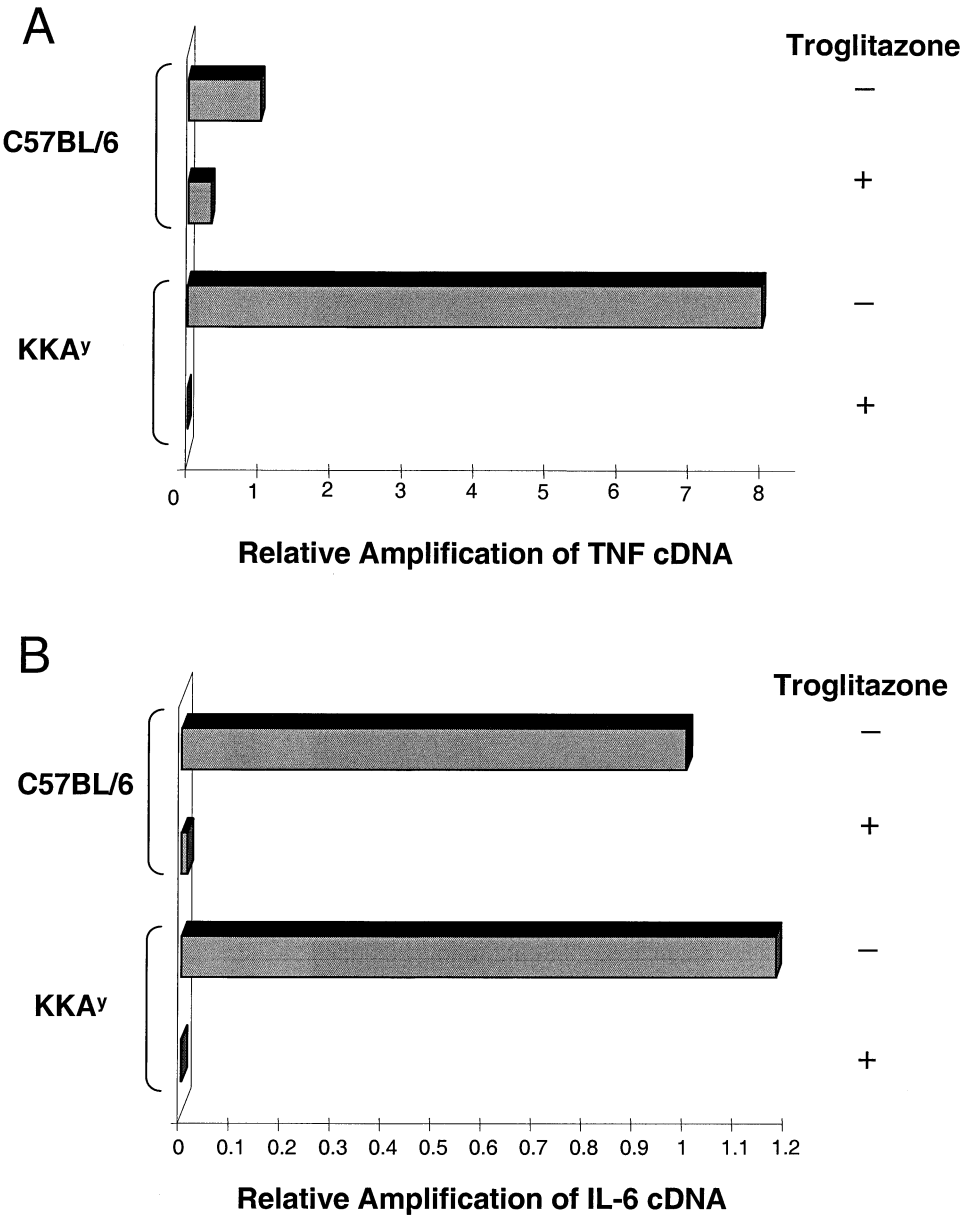


**FIG. 5. Effects of troglitazone on LPS-stimulated TNF-α (A) and IL-6 (B) expression in white adipose tissue of lean C57BL6 and obese KKA$^y$ mice. Total adipose tissue RNA from vehicle- or troglitazone-treated mice was isolated 90 min posttreatment with LPS (2.5 μg/g), reverse-transcribed, and amplified with TNF-α or IL-6-specific primers. PCR products were incubated with SYBR Green, and the number of cycles necessary to detect a fluorescence signal was determined. Relative alterations in mRNA expression were calculated from these kinetic data and expressed as change relative to vehicle-treated lean control mice. Data are values of pooled cDNA from 3 individual mice.**

of the expression level present in adipose tissue [28]. The increased levels of circulating fatty acids, which occur under the conditions of NIDDM, have been suggested to serve as specific activators of the γ2 promoter in a tissue not normally expressing this isoform [28]. Alternatively, it is possible that, in addition to PPARγ, other pathways may also determine the troglitazone-induced negative regulation of TNF-α and IL-6 in the liver. Commensurate with this latter hypothesis is the observation that rosiglitazone, a structurally related TZD featuring an approximately 100-fold higher affinity for PPARγ than troglitazone [29], did not down-regulate TNF-α gene expression at a pharmacologically active concentration in KKA$^y$ mice.* Thus, the compound specificity of these effects has to be further analyzed.

Our observation that troglitazone down-regulates TNF-α and IL-6 in liver is provocative, because it suggests that this treatment may render the liver refractory to stimulating effects caused by agents that trigger the release of TNF-α. Indeed, one of the most effective stimulators of TNF-α biosynthesis and release, bacterial LPS, was largely ineffective in troglitazone-treated obese KKA$^y$ mice. LPS exerts its

* Bedoucha M and Boelsterli UA, unpublished observations.

effects on Kupffer cells by mechanisms that are different from other target cells. For example, Kupffer cells do not express CD 14, the receptor for the LPS/LPS-binding protein complex [30]. Nevertheless, LPS activates Kupffer cells to release proinflammatory cytokines including TNF-α. It has been widely accepted that Kupffer cells are the major source of TNF-α after LPS challenge [31]. Besides the soluble form of TNF-α secreted into the bloodstream, a membrane-associated form of TNF-α exists which is involved in local (paracrine) reactions [32]. In this study, it was most likely the membrane-bound TNF-α that was detected in liver homogenates of mice challenged with LPS. It is also this membrane-associated form that has been implicated in mediating the hepatic toxicity of LPS [33]. Thus, it is possible that troglitazone may attenuate the cytotoxic action of liver-associated TNF-α, which can trigger the caspase activation cascade leading to apoptosis in sensitized hepatocytes [34].

In contrast, besides inducing hepatocyte death, the pleiotropic action of TNF-α includes tissue-rescuing pathways, such as cell proliferation in the liver [35–38]. Therefore, in view of the pivotal roles of both TNF-α and IL-6 in hepatic tissue regeneration, the troglitazone-induced down-regulation of TNF-α and related cytokines may interfere with some of these processes. Because tissue regeneration after a subtoxic chemical insult is crucial for the structural and functional integrity of the liver [39–41], sustained inhibition of the expression of a number of cytokines involved in these regenerative processes may have unfavorable consequences.

Indeed, increasing evidence indicates that the clinical use of troglitazone is associated with rare but severe cases of hepatic adverse effects which have resulted in several deaths and a number of liver transplants [16, 42–44]. The mechanism underlying troglitazone-induced idiosyncratic liver injury has not been elucidated. In particular, the relationship of hepatic toxicity with a possible disruption of the cytokine network remains speculative. Nevertheless, the massive down-regulation by troglitazone of hepatic TNF-α and IL-6, as observed in this murine model of NIDDM, if uncompensated could result in a dual effect. On the one hand, the lack of responsiveness towards inducers of hepatic TNF-α and IL-6 production may play a beneficial role in decreasing extrahepatic TNF-α levels and in ameliorating tissue insulin resistance. Conversely, this negative regulation of selective cytokines may disrupt the TNF-α and IL-6-mediated regulatory defense mechanisms and signaling pathways involved in the acute phase response and tissue remodeling following toxic liver injury.

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