

# Mechanism of the Anti-Inflammatory Effect of Thiazolidinediones: Relationship with the Glucocorticoid Pathway

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## ABSTRACT

The glucocorticoid receptor (GR) and peroxisome proliferator-activated receptors (PPARs) play important roles in both physiological and pathological conditions such as cell differentiation, lipolysis, control of glucose metabolism, immunity, and inflammation. In fact, recent studies suggest that the thiazolidinedione (TZD) class of PPAR- $\gamma$  ligands, like glucocorticoids, may also be clinically beneficial in several inflammatory diseases, even if the molecular mechanisms responsible for these activities have not yet been clarified. In this study, by using a murine model of inflammation, the carrageenin-induced paw edema in mouse, we show that the anti-inflammatory activity exhibited by the PPAR- $\gamma$  agonists rosiglitazone and ciglitazone

is reversed by the GR antagonist RU486 (17 $\beta$ -hydroxy-11 $\beta$ -[4-dimethylamino phenyl]-17 $\alpha$ -[1-propynyl]estra-4,9-dien-3-one). Moreover, by using a conditional GR null cell line, we demonstrate, for the first time to our knowledge, that one of the possible mechanisms explaining the anti-inflammatory activity of TZDs is their ability to activate GR nuclear translocation. In addition, by using J774 cell line lacking PPAR- $\gamma$ , we demonstrate that PPAR- $\gamma$  expression could not be essential for TZD-mediated GR nuclear translocation, thus explaining, at least in part, the molecular mechanism underlying their anti-inflammatory activity.

Peroxisome proliferator-activated receptor (PPAR)- $\gamma$  is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors that are related to retinoid, steroid, and thyroid hormone receptors (Evans, 1988). All members of this superfamily have a similar structural organization: an N-terminal region that allows ligand-independent activation (Werman et al., 1997) followed by a DNA-binding domain and the C-terminal ligand-binding domain (Moras and Gronemeyer, 1998). The PPAR family consists of three subtypes: PPAR- $\alpha$ , PPAR- $\delta$  (also known as NUC1), and PPAR- $\gamma$  (Lemberger et al., 1996). PPAR- $\gamma$  has been suggested to be involved in a broad range of cellular functions, including adipocyte differentiation (Spiegelman and Flier, 1996), glucose homeostasis (Deeb et al., 1998), inflammatory response (Jiang et al., 1998; Ricote et al., 1998), and apoptosis (Chinetti et al., 1998). This receptor is the molecular target of

fatty acid derivatives, the thiazolidinedione (TZD) class of antidiabetic drugs, which includes rosiglitazone and ciglitazone and certain nonsteroidal anti-inflammatory drugs (Schoonjans et al., 1997; Willson et al., 2000). Recent studies suggest that the TZD class of PPAR- $\gamma$  ligands may also be clinically beneficial in inflammatory bowel disease (Ma et al., 1998). In fact, there has recently been considerable interest in the role of PPAR- $\gamma$  in regulating the inflammatory response, because 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> and other PPAR- $\gamma$  agonists inhibit the expression of a variety of proteins with proinflammatory properties, including cyclooxygenase-2 (COX-2), inducible nitric-oxide synthase (iNOS), and several cytokines (Daynes and Jones, 2002). However, the molecular mechanisms responsible for these activities have not yet been clarified. In fact, it is important to point out that the anti-inflammatory activity seen with rosiglitazone occurred at concentrations considerably higher than the  $K_d$  value for binding PPAR- $\gamma$  or the concentration needed to elicit adipogenesis and insulin sensitization. Thus, a role for

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**ABBREVIATIONS:** PPAR, peroxisome proliferator-activated receptor; TZD, thiazolidinedione; COX, cyclooxygenase; iNOS, inducible nitric-oxide synthase; GC, glucocorticoid; GR, glucocorticoid receptor; IL, interleukin; LPS, lipopolysaccharide; RU486, 17 $\beta$ -hydroxy-11 $\beta$ -[4-dimethylamino phenyl]-17 $\alpha$ -[1-propynyl]estra-4,9-dien-3-one; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; RT-PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction; IFN, interferon; BADGE, bisphenol A diglycidyl ether; Dex, dexamethasone; CREB, cAMP response element-binding protein.

the receptor in mediating the anti-inflammatory activity of PPAR- $\gamma$  ligands is not ensured. Moreover, it has been recently shown that TZDs exert anti-inflammatory effects in macrophages PPAR- $\gamma$  (-/-), indicating that their anti-inflammatory activity is not only related to PPAR- $\gamma$  (Chawla et al., 2001).

Glucocorticoids (GCs) play a key role in regulating diverse physiological processes, such as metabolism, salt and water balance, cell proliferation, differentiation, inflammation, and immune response (Newton, 2000). Their effects are exerted by binding to the intracellular glucocorticoid receptor (GR), which belongs, as does PPAR- $\gamma$ , to the nuclear receptor gene family (Willson et al., 2000). Steroid hormones regulate the transcription of numerous genes via high-affinity receptors that act in concert with chromatin remodeling complexes, coactivators, and corepressors, among which steroid receptor coactivator 1 plays an important role (Feng et al., 1998). In fact, GCs can down-regulate the expression of interleukin (IL)-6 and iNOS both induced by various inflammatory stimuli such as lipopolysaccharide (LPS) (Caldenhoven et al., 1995).

In this study, we investigated the possibility of an interaction between TZDs and GR signaling pathway. We show that the anti-inflammatory activity exhibited by the PPAR- $\gamma$  agonists rosiglitazone and ciglitazone is reversed by the GR antagonist RU486, by using a murine model of inflammation, the carrageenin-induced paw edema in mouse. Moreover, our in vitro results demonstrate, for the first time to our knowledge, that the anti-inflammatory activity of TZDs is a result, at least in part, of their ability to activate GR nuclear translocation independently from PPAR- $\gamma$ .

## Materials and Methods

**Cell Culture.** E8.2 cells, derived from mouse L929 fibroblasts (Housley and Forshoefel, 1989), are spontaneous glucocorticoid-resistant cells and contain neither detectable GR protein nor mRNA transcripts, whereas in E8.2/GR3 cells the GR protein levels are reconstituted and are regulated by tetracycline both temporally and in a dose-dependent manner as shown previously (Wei et al., 1998).

GR null mouse fibroblast cells E8.2 were maintained in 175-cm<sup>2</sup> flasks in Dulbecco's modified Eagle's medium (DMEM) (Cambrex Bio Science Verviers S.p.a., Verviers, Belgium) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. E8.2/GR3 cells were grown in DMEM supplemented with 10% FCS, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 200  $\mu$ g/ml of G418 (Geneticin; Invitrogen, Milan, Italy), 200  $\mu$ g/ml hygromycin B (Invitrogen), and 1  $\mu$ g/ml tetracycline (Sigma, Milan, Italy).

The murine monocyte/macrophage cell line J774 was from the European Collection of Animal Cell Cultures (Salisbury, UK). J774 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 5 mM sodium pyruvate. All the cells were grown at 37°C in a humidified incubator under 5% CO<sub>2</sub>.

**RT-PCR of IL-6, iNOS, and PPAR- $\gamma$ .** Total RNA was isolated from the cell using TRIzol (Invitrogen). In brief, the cells were washed twice with ice-cold PBS, and then 1 ml of TRIzol reagent was added to each 10-cm dish. The cells were collected after scraping, transferred to a microcentrifuge tube, and homogenized by passing 5 to 10 times in 20-gauge needle fitted onto 3-ml syringe.

Chloroform (200  $\mu$ l) was added, and the tube was shaken for 15 s, followed by centrifugation at 12,000g for 15 min. The aqueous phase was transferred to a new microcentrifuge tube, and the total RNA was precipitated using 0.5 ml of isopropyl alcohol. RNA was allowed

to precipitate at room temperature for 10 min and centrifuged at 12,000g for 10 min. The supernatant was removed, and the RNA pellet was washed with 1 ml of 70% ethanol followed by centrifugation at 7500g for 5 min. The RNA pellet was air-dried for 5 min, resuspended in diethyl pyrocarbonate-treated water and then heated at 55°C for 15 min. The final amount of RNA was determined by absorbance at 260 nm. Then, 7  $\mu$ g of total RNA was reverse-transcribed into cDNA by using oligo(dT)<sub>12-18</sub> primer (Invitrogen) and Superscript II Reverse Transcriptase (Invitrogen). One microliter of cDNA was amplified by PCR using *Taq* Polymerase (Invitrogen) according to the manufacturer's instructions. The primers were iNOS: sense, 5'-TGGAATGGAGACTGTCCCAG3', and antisense, 5'-GGGATCTGAATGTGATGTTTG-3'; IL-6: sense, 5'-GGTCTTCTGAGTACCATAGCTAC-3' and antisense, 5'-GGAATGTCCACAACTGATATGC-3'; PPAR- $\gamma$ : sense, 5'-AAAGATGGAGTCTCATCTC-3' and antisense, 5'-AGCAGGGGGTGAAGGCTCAT-3' (Nakamichi et al., 2003); and  $\beta$ -ACTIN: sense, 5'-ATGAAGATCCTGACCGCGCT-3' and antisense, 5'-AACGCAGCTCAGTAACAGTCCG-3'. The amplified fragments were 305, 327, 400, and 584 base pairs, respectively.

The PCR reaction was performed under the following conditions: a first cycle of denaturation at 94°C for 1 min 40 s and then 25 or 30 cycles of denaturation at 94°C for 40 s, annealing at 54°C (PPAR- $\gamma$ ) or 56°C (all others) for 40 s, extension at 72°C for 1 min, and one additional cycle of extension at 72°C for 8 min. The PCR products were run on a 1% agarose gel and visualized by ethidium bromide staining.

**Assay for Cytokines.** IL-6 levels in the cell culture medium were assayed by using a commercially available mouse cytokine enzyme-linked immunosorbent assay test kits (Pierce Endogen, Rockford, IL) according to the manufacturer's instructions, and the results expressed as nanograms per milliliter and represent the mean  $\pm$  S.E.M. of *n* experiments run in triplicate.

**NO<sub>2</sub><sup>-</sup> Assay.** The amount of NO<sub>2</sub><sup>-</sup>, stable metabolite of nitric oxide, present in culture media from cells was measured 24 h after LPS from *Escherichia coli* (Fluka, Milan, Italy) with or without IFN- $\gamma$  stimulation, with the Griess reaction as described previously (Ianaro et al., 2000). Results are expressed as nanomoles per milliliter and represent the mean  $\pm$  S.E.M. of *n* experiments run in triplicate.

**Preparation of Nuclear Extracts.** All the extraction procedures were performed on ice with ice-cold reagents. Stimulated or unstimulated E8.2/GR3 and J774 cells were washed twice with ice-cold PBS and centrifuged at 1500g for 10 min at 4°C. The cell pellet was resuspended in one packed cell volume of lysis buffer and incubated on ice for 5 min with occasional vortexing. After centrifugation at 1500g at 4°C for 5 min, one cell pellet volume of extraction buffer was added to the nuclear pellet and incubated on ice for 15 min with occasional vortexing. Nuclear proteins were isolated by centrifugation at 13,000g for 15 min, and the supernatant was aliquoted and stored at -80°C. Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad, Milan, Italy).

**Western Blot Analysis.** Immunoblotting analysis of GR and actin proteins was performed on nuclear cell extracts. Equivalent amounts of protein (65  $\mu$ g) from each sample were electrophoresed in an 8% discontinuous polyacrylamide minigel. The proteins were transferred onto nitrocellulose membranes according to the manufacturer's instructions (Bio-Rad). The membranes were saturated by incubation with 10% nonfat dry milk in PBS-0.1% Triton X-100 for 3 h at room temperature and then incubated with anti-GR mouse antibody (1:200) (Affinity Bioreagents, Golden, CO) or anti-actin (1:300) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) goat antibody overnight at 4°C. The membranes were washed three times with 0.1% Tween 20 in PBS and then incubated with anti-mouse or anti-goat (1:1000) immunoglobulins coupled to peroxidase (DakoCytomation, Milan, Italy) for 1 h at room temperature. The immune complexes were visualized by the enhanced chemiluminescence method (Amersham Biosciences Inc., Cologno Monzese, Italy). Thereafter, the relative presence of GR and actin was quantified by densitometric scanning of the X-ray films with GS-700 imaging den-

sitometer (Bio-Rad) and a computer program (Molecular Analyst; IBM, White Plains, NY).

**GR Binding Assay.** The binding assay described by Cheron et al. (2004) was used, with minor modifications. In brief, J774 cells ( $1 \times 10^6$ /ml) were incubated in culture medium supplemented with 2.5% FCS and containing [ $^3$ H]dexamethasone (specific activity, 88 Ci/mmol; Amersham Biosciences Inc.) for 2 h at 37°C. TZD treatments were performed 1 h before dexamethasone. After incubation, monolayers were washed six times with ice-cold PBS, and cells were lysed in 1 N NaOH. Lysates were harvested and counted in a beta spectrometer. Bound [ $^3$ H]dexamethasone was quantified by liquid scintillation, and the specific concentration was calculated by subtracting the nonspecific binding (determined with a 1000-fold excess cold dexamethasone). Scatchard plot analysis was performed to determine the dissociation constant  $K_d$  and the maximal number of binding sites ( $B_{max}$ ) values using a concentration range of 1 to 32 nM [ $^3$ H]dexamethasone. For one-point binding assays, 10 nM [ $^3$ H]dexamethasone was used.

**Animals.** Male ICR mice (Harlan, Milan, Italy), weighing 25 to 30 g, were used in all experiments. Animals were provided with food and water ad libitum. The light cycle was automatically controlled (on 7:00 AM, off 7:00 PM), and the room temperature was thermostatically regulated to  $22 \pm 1^\circ\text{C}$ . Before the experiments, animals were housed in these conditions for 3 to 4 days to become acclimated. Animal care was in accordance with Italian and European regulations on protection of animals used for experimental and other scientific purposes.

**Paw Edema.** Paw edema was induced by subplantar injection of 50  $\mu\text{l}$  of sterile saline containing 1%  $\lambda$ -carrageenin into the right hind paw. Paw volumes were measured by a plethysmometer (Ugo Basile, Milan, Italy) at varying time intervals. The increase in paw volume was evaluated as difference between the paw volume measured at each time point and the basal paw volume measured immediately before carrageenin injection.

**Treatments.** The test agents used in this study were rosiglitazone (0.1–3 mg/kg i.p.), ciglitazone (3 mg/kg i.p.) bisphenol A diglycidyl ether (BADGE, 10 mg/kg s.c.), RU486 (10 mg/kg i.p.), dexamethasone (Dex; 0.06–0.125 mg/kg i.p.), and actinomycin D (0.5 mg/kg i.p.). Rosiglitazone, ciglitazone, BADGE, and RU486 were given 1 h before carrageenin injection and every 24 h thereafter. Dexamethasone was given 2 h before carrageenin injection and every 24 h thereafter. Actinomycin D was administered only 1 h before subplantar injection of carrageenin.

**Statistical Analysis.** Values are expressed as the mean  $\pm$  S.E.M. of  $n$  animals for in vivo experiments and of  $n$  experiments run in triplicate for in vitro experiments. Comparisons were calculated by one-way analysis of variance and Bonferroni-corrected  $p$  value for multiple comparisons. The level of statistically significant difference was defined as  $p < 0.05$ .

## Results

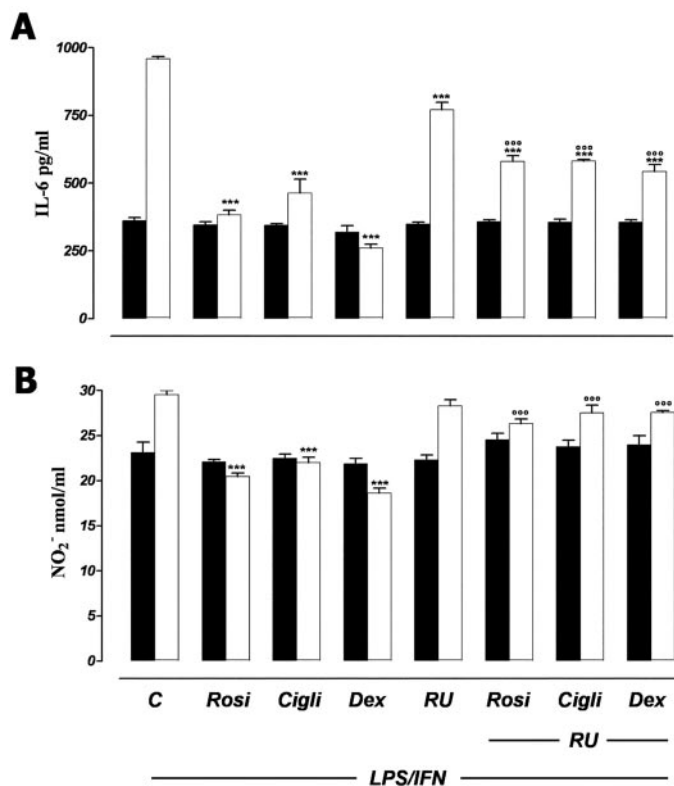
### In Vitro

**Effect of Rosiglitazone, Ciglitazone, Dexamethasone, and RU486 on IL-6 Production and IL-6 mRNA Expression Levels by E8.2 and E8.2/GR3 Cells.** In preliminary experiments, we established that cell viability ( $>95\%$ ) was not affected by any of the treatments (data not shown).

The production of IL-6 by unstimulated E8.2 or by E8.2/GR3 cells was undetectable ( $<15$  pg/ml;  $n = 6$ ). Incubation of E8.2 cells with a combination of 5  $\mu\text{g}/\text{ml}$  LPS and 100 U/ml IFN- $\gamma$  for 24 h caused a release of IL-6 ( $367.6 \pm 17$  pg/ml). Neither 10  $\mu\text{M}$  rosiglitazone, 10  $\mu\text{M}$  ciglitazone, nor 1  $\mu\text{M}$  dexamethasone modified LPS/IFN-induced IL-6 release (Fig. 1). Stimulation of E8.2/GR3 cells with the same combination

of LPS/IFN for 24 h induced a substantial increase of IL-6 production compared with E8.2 cells ( $940 \pm 8.8$  pg/ml). We were surprised to find that 10  $\mu\text{M}$  rosiglitazone, 10  $\mu\text{M}$  ciglitazone, and 1  $\mu\text{M}$  dexamethasone all significantly ( $p < 0.001$ ) inhibited LPS/IFN-induced IL-6 release by 60, 52, and 73%, respectively; more interestingly, this inhibition was significantly ( $p < 0.001$ ), albeit only partially, reversed by 400 nM RU486 (Fig. 1). In dose-response experiments, 1 to 0.1  $\mu\text{M}$  rosiglitazone inhibited IL-6 production by 25% ( $p < 0.05$ ) and 5%, respectively (data not shown). Similar results were obtained in experiments carried out in serum-free medium, to verify that potential serum glucocorticoids do not cooperate for TZD anti-inflammatory effect (data not shown).

Stimulation of E8.2 or E8.2/GR3 cells with a combination of 5  $\mu\text{g}/\text{ml}$  LPS and 100 U/ml IFN- $\gamma$  for 6 h caused a significant increase of IL-6 mRNA expression levels compared with unstimulated cells (Fig. 2A). Neither 10  $\mu\text{M}$  rosiglitazone, 10  $\mu\text{M}$  ciglitazone, nor 1  $\mu\text{M}$  dexamethasone, all preincubated 2 h before LPS/IFN stimulation, modified LPS/IFN-induced IL-6 mRNA expression in E8.2 cells (Fig. 2A). In contrast, 10  $\mu\text{M}$  rosiglitazone, 10  $\mu\text{M}$  ciglitazone, and 1  $\mu\text{M}$  dexamethasone almost completely inhibited LPS/IFN-induced IL-6 mRNA ex-



**Fig. 1.** Effect of rosiglitazone, ciglitazone, and dexamethasone on IL-6 and nitrite production in E8.2 and E8.2/GR3 cells. GR null mouse fibroblast E8.2 and GR reconstituted mouse fibroblast E8.2/GR3 cells were suspended in DMEM (10% FCS) at  $2.5 \times 10^5$  cells/ml. Cells were stimulated with a combination of 5  $\mu\text{g}/\text{ml}$  LPS and 100 U/ml IFN- $\gamma$  for 24 h. Rosiglitazone (Rosi; 10  $\mu\text{M}$ ), 10  $\mu\text{M}$  ciglitazone (Cigli), and 1  $\mu\text{M}$  Dex were all preincubated 2 h before LPS/IFN stimulation. RU486 (RU; 400 nM) was added 2 h before rosiglitazone or ciglitazone or dexamethasone treatment. A, effect of TZDs and dexamethasone on IL-6 production in E8.2 (filled columns) and E8.2/GR3 (open columns) cells. B, effect of TZDs and dexamethasone on nitrite production in E8.2 (filled columns) and E8.2/GR3 (open columns) cells. Data shown are from three independent experiments and are expressed as mean  $\pm$  S.E.M. C, LPS/IFN-stimulated cells. \*\*\*,  $p < 0.001$  versus C; \*\*\*,  $p < 0.001$  versus Rosi, Cigli, or Dex without RU.



pression levels in E8.2/GR3; more interestingly, this inhibition was, albeit only partially, reversed when the E8.2/GR3 were prechallenged with 400 nM RU486 2 h before rosiglitazone, ciglitazone, or dexamethasone treatment (Fig. 2A).

Densitometric analysis of IL-6 mRNA levels in E8.2/GR3, normalized to expression levels of housekeeping gene  $\beta$ -actin (Fig. 2B), revealed a significant ( $p < 0.001$ ) inhibition of LPS/IFN-induced IL-6 mRNA expression in cells treated

with 10  $\mu$ M rosiglitazone, 10  $\mu$ M ciglitazone, or 1  $\mu$ M dexamethasone by 68, 60, or 88%, respectively (Fig. 2B). Prechallenge with 400 nM RU486 caused a partial but significant ( $p < 0.001$ ) reversion of this inhibition.

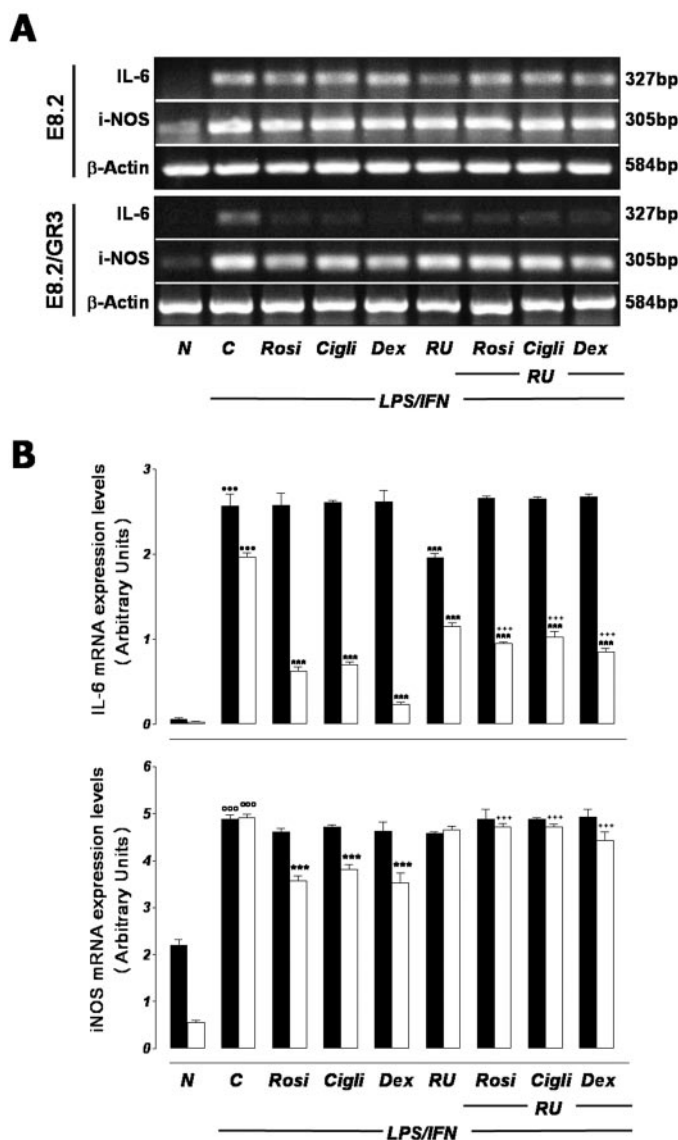
**Effect of Rosiglitazone, Ciglitazone, Dexamethasone, and RU486 on  $\text{NO}_2^-$  Production and iNOS mRNA Expression Levels by E8.2 and E8.2/GR3 Cells.** The production of  $\text{NO}_2^-$  by unstimulated E8.2 and E8.2/GR3 cells was undetectable ( $<5$  nmol/ml;  $n = 4$ ). Incubation of the cells with LPS/IFN for 24 h caused a substantial release of  $\text{NO}_2^-$  ( $23 \pm 1.1$  nmol/ml;  $n = 9$ ). Stimulation of E8.2 cells with LPS/IFN in presence of either 10  $\mu$ M rosiglitazone, 10  $\mu$ M ciglitazone, or 1  $\mu$ M dexamethasone did not modify  $\text{NO}_2^-$  release (Fig. 1). Stimulation of E8.2/GR3 cells with LPS/IFN caused a substantial release of  $\text{NO}_2^-$  ( $29.5 \pm 0.77$  nmol/ml) compared with unstimulated cells. As already shown for IL-6 release, 10  $\mu$ M rosiglitazone, 10  $\mu$ M ciglitazone, and 1  $\mu$ M dexamethasone significantly ( $p < 0.001$ ) inhibited LPS/IFN induced  $\text{NO}_2^-$  release by 31, 26, and 37%, respectively (Fig. 1). When cells were stimulated with LPS/IFN in the presence of 400 nM RU486, 10  $\mu$ M rosiglitazone, 10  $\mu$ M ciglitazone, and 1  $\mu$ M dexamethasone, a reversion of the inhibitory effects exhibited by all drugs was observed (Fig. 1). In dose-response experiments, 1  $\mu$ M rosiglitazone inhibited  $\text{NO}_2^-$  production by 17% ( $p < 0.05$ ), whereas 0.1  $\mu$ M rosiglitazone did not modify  $\text{NO}_2^-$  production compared with that observed in control cells (data not shown).

Stimulation of E8.2 or by E8.2/GR3 cells with a combination of 5  $\mu$ g/ml LPS and 100 U/ml IFN- $\gamma$  for 6 h caused a significant increase of iNOS mRNA expression levels compared with those of unstimulated cells (Fig. 2A). Neither 10  $\mu$ M rosiglitazone, 10  $\mu$ M ciglitazone, nor 1  $\mu$ M dexamethasone, all preincubated 2 h before LPS/IFN stimulation, modified LPS/IFN-induced iNOS mRNA expression in E8.2 cells. In contrast, 10  $\mu$ M rosiglitazone, 10  $\mu$ M ciglitazone, and 1  $\mu$ M dexamethasone significantly ( $p < 0.001$ ) inhibited LPS/IFN-induced iNOS mRNA expression levels in E8.2/GR3; more interestingly, this inhibition was reversed when the E8.2/GR3 were prechallenged with 400 nM RU486 2 h before rosiglitazone, ciglitazone, or dexamethasone treatment (Fig. 2A).

Densitometric analysis of iNOS mRNA levels in E8.2/GR3, normalized to expression levels of housekeeping gene  $\beta$ -actin (Fig. 2B), revealed a significant ( $p < 0.001$ ) inhibition of LPS/IFN-induced iNOS mRNA expression in cells treated with 10  $\mu$ M rosiglitazone, 10  $\mu$ M ciglitazone, or 1  $\mu$ M dexamethasone by 28, 22, or 29%, respectively (Fig. 2B), whereas the prechallenge with 400 nM RU486 reversed the inhibitory effect.

**Effect of Rosiglitazone, Ciglitazone, and Dexamethasone on GR Nuclear Translocation in E8.2/GR3 Cells.** Intriguingly, TZDs as well as dexamethasone induced nuclear translocation of GR in E8.2/GR3 cells, as clearly shown by Western blot analysis and relative densitometric analysis (Fig. 3).

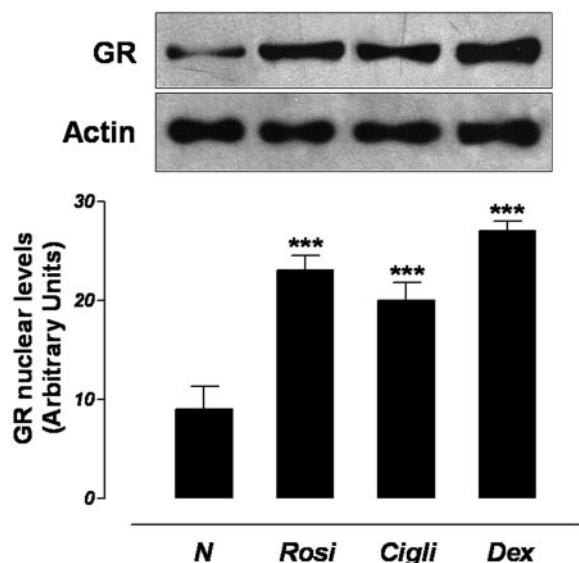
**RT-PCR of PPAR- $\gamma$ .** To verify whether E8.2 cells and E8.2/GR3 cells expressed PPAR- $\gamma$ , an RT-PCR was carried out. As shown in Fig. 4A, both cell lines expressed PPAR- $\gamma$  mRNA that was not modified by LPS/IFN or rosiglitazone challenge. In contrast, murine macrophages J774 does not express PPAR- $\gamma$  mRNA (Chawla et al., 2001) also after LPS or rosiglitazone stimulation, as shown by RT-PCR (Fig. 4B). Therefore, we used this cell line to test the role (if any) of PPAR- $\gamma$  on TZD anti-inflammatory effect (see below).



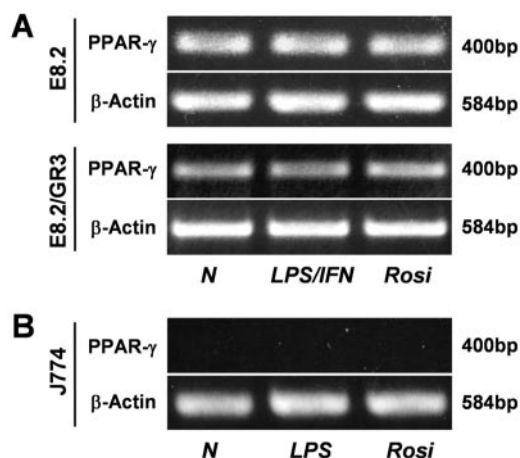
**Fig. 2.** Effect of rosiglitazone, ciglitazone, and dexamethasone on IL-6 and iNOS mRNA expression in E8.2 and E8.2/GR3 cells. GR null mouse fibroblast E8.2 and GR reconstituted fibroblast E8.2/GR3 cells were suspended in DMEM (10% FCS) at  $1.0 \times 10^6$  cells/ml. Cells were stimulated with a combination of 5  $\mu$ g/ml LPS and 100 U/ml IFN- $\gamma$  for 6 h. Rosiglitazone (Rosi; 10  $\mu$ M), 10  $\mu$ M ciglitazone (Cigli), and 1  $\mu$ M Dex were all preincubated 2 h before LPS/IFN stimulation. RU486 (RU; 400 nM) was added 2 h before rosiglitazone, ciglitazone, or dexamethasone treatment. A, 30 cycles of PCR reaction of reverse-transcribed mRNA into cDNA were performed by using specific primers for IL-6, iNOS, and  $\beta$ -actin as described under *Materials and Methods*. B, densitometric analysis of mRNA expression of IL-6 (top) and iNOS (bottom) in both E8.2 (filled columns) and E8.2/GR3 cells (open columns) normalized to expression levels of housekeeping gene  $\beta$ -actin. Data shown are from three independent experiments and are expressed as mean  $\pm$  S.E.M. N, unstimulated cells; C, LPS/IFN-stimulated cells.  $^{\circ\circ}$ ,  $p < 0.001$  versus N;  $^{***}$ ,  $p < 0.001$  versus C;  $^{+++}$ ,  $p < 0.001$  versus Rosi, Cigli, or Dex without RU.

**Effect of Rosiglitazone, Ciglitazone, Dexamethasone, and of RU486 on IL-6 Production and IL-6 mRNA Expression Levels by J774 Cells.** In preliminary experiments, we established that cell viability (>95%) was not affected by any of the treatments (data not shown).

The production of IL-6 by unstimulated J774 cells was



**Fig. 3.** Effect of rosiglitazone, ciglitazone, and dexamethasone on GR nuclear translocation in E8.2/GR3 cells. GR reconstituted mouse fibroblast E8.2/GR3 cells were suspended in DMEM (10% FCS) at  $1 \times 10^6$  cells/ml. Cells were stimulated with rosiglitazone (Rosi; 10  $\mu$ M), 10  $\mu$ M ciglitazone (Cigli), and 1  $\mu$ M Dex for 2 h. Thereafter nuclear extracts were prepared as described under *Materials and Methods*. Western blot analysis for GR protein was performed as described under *Materials and Methods* (top), and equal loading was confirmed by actin staining. Densitometric analysis of GR nuclear levels (bottom). Data shown are from three independent experiments and are expressed as mean  $\pm$  S.E.M. N, unstimulated cells. \*\*\*,  $p < 0.001$  versus N.



**Fig. 4.** RT-PCR of PPAR- $\gamma$ . A, GR null mouse fibroblast E8.2 and GR reconstituted mouse fibroblast E8.2/GR3 cells were suspended in DMEM (10% FCS) at  $1 \times 10^6$  cells/ml. Cells were stimulated with a combination of 5  $\mu$ g/ml LPS and 100 U/ml IFN- $\gamma$  (LPS/IFN) or 10  $\mu$ M rosiglitazone (Rosi) for 6 h. Twenty-five cycles of PCR reaction of reverse-transcribed mRNA into cDNA were performed by using specific primers for PPAR- $\gamma$  and  $\beta$ -actin as described under *Materials and Methods*. N, unstimulated cells. B, murine macrophages J774 were plated in DMEM (10% FCS) at  $1 \times 10^6$  cells/ml. Cells were stimulated with 100 ng/ml LPS or 10  $\mu$ M Rosi for 6 h. Thirty cycles of PCR reaction of reverse-transcribed mRNA into cDNA were performed by using specific primers for PPAR- $\gamma$  and  $\beta$ -actin as described under *Materials and Methods*. N, unstimulated cells.

undetectable ( $<15$  pg/ml;  $n = 6$ ). Incubation of J774 cells with 0.1  $\mu$ g/ml LPS for 24 h caused a significant release of IL-6 ( $5900 \pm 320$  pg/ml). In dose-response experiments, 0.1 to 10  $\mu$ M rosiglitazone inhibited IL-6 production by 2, 15 ( $p < 0.01$ ), and 37% ( $p < 0.001$ ), respectively (Fig. 5A). Ciglitazone (10  $\mu$ M) and dexamethasone (1  $\mu$ M) significantly ( $p < 0.001$ ) inhibited LPS-induced IL-6 release by 30 and 50%, respectively (data not shown). Similar results were obtained in experiments carried out in serum-free medium, to verify that potential serum glucocorticoids do not cooperate for TZD anti-inflammatory effect (data not shown).

Stimulation of J774 cells with 0.1  $\mu$ g/ml LPS for 6 h caused a significant increase of IL-6 mRNA expression levels compared with unstimulated cells (Fig. 5B). Rosiglitazone (10  $\mu$ M), 10  $\mu$ M ciglitazone, and 1  $\mu$ M dexamethasone, all preincubated 2 h before LPS stimulation, inhibited significantly LPS-induced IL-6 mRNA expression levels by 18 ( $p < 0.001$ ), 15 ( $p < 0.01$ ), and 33% ( $p < 0.001$ ), respectively. It is interesting that this inhibition was significantly ( $p < 0.001$ ) reversed when J774 were prechallenged with 400 nM RU486 30 min before rosiglitazone, ciglitazone, or dexamethasone treatment (Fig. 5B).

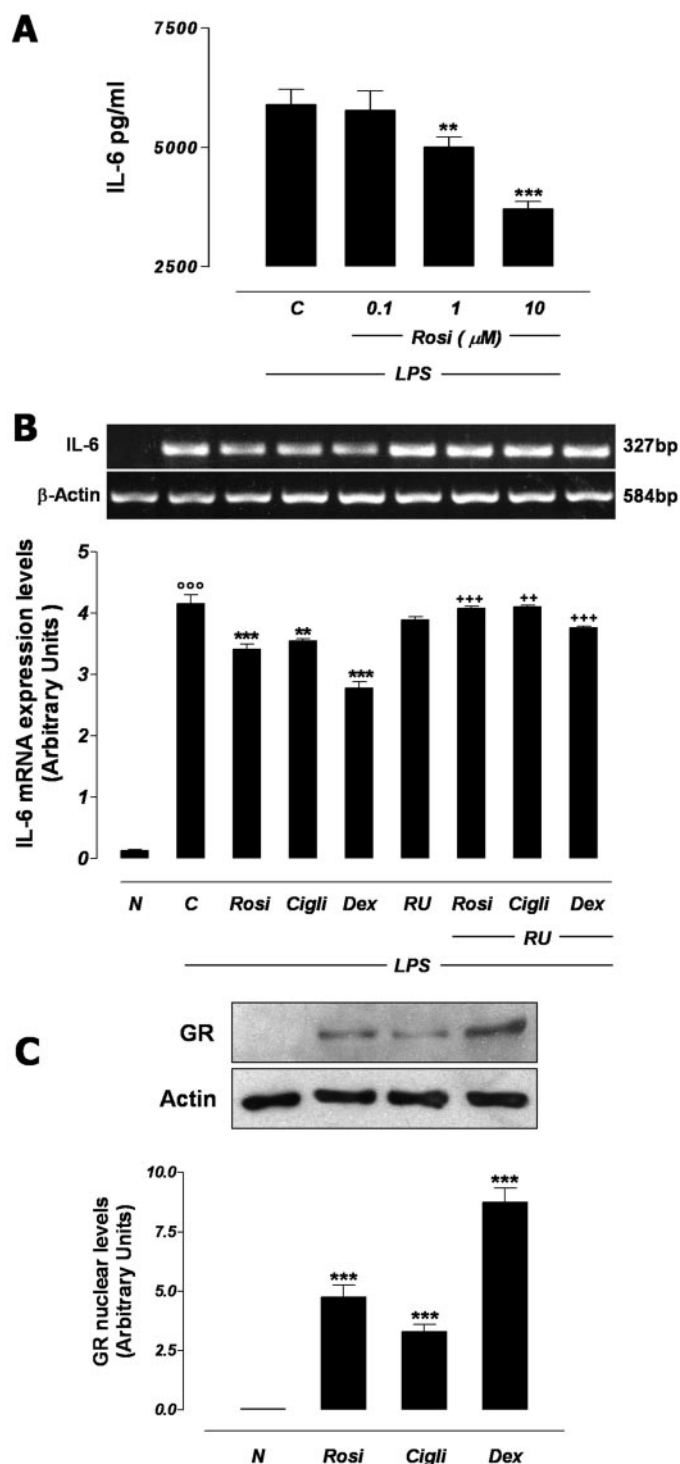
**Effect of Rosiglitazone, Ciglitazone, and Dexamethasone on GR Nuclear Translocation in J774 Cells.** It is interesting that, also in J774 cells, TZDs and dexamethasone induced nuclear translocation of GR, as shown by Western blot analysis (Fig. 5C), suggesting that PPAR- $\gamma$  could be not essential for TZDs to exert anti-inflammatory effects.

**Effect of Rosiglitazone and Ciglitazone on Dexamethasone-GR Binding in J774 Cells.** J774 cells displayed an avid binding to [ $^3$ H]dexamethasone ( $K_d = 17.08 \pm 1.16$  nM;  $B_{max} = 23.73 \pm 0.26$  pM from three experiments). Exposure of J774 cells to 0.1 to 10  $\mu$ M rosiglitazone did not significantly affect [ $^3$ H]dexamethasone-specific binding to GR (Fig. 6). Similar results were obtained with ciglitazone (data not shown).

## In Vivo

**Effect of Rosiglitazone and Ciglitazone on Mouse Paw Edema.** Carrageenin injection into the subplantar area caused a time-dependent increase of paw volume in mice. This edema developed along two distinct phases: an acute first phase peaking at 5 h and a second phase peaking at 72 h (Fig. 7). Treatment of animals with rosiglitazone (0.1, 1, and 3 mg/kg i.p.), before and after carrageenin injection, reduced paw edema in a dose-dependent manner throughout the time course of the edema. Thus, at the time of maximal foot increase during the first phase (5 h), 1 and 3 mg/kg rosiglitazone inhibited the inflammatory reaction by 22 ( $p < 0.01$ ) and 42% ( $p < 0.01$ ), respectively, whereas 0.1 mg/kg did not modify edema formation. A similar profile of activity was also observed at 72 h (Fig. 7). As shown in Fig. 7, inset, ciglitazone given at 3 mg/kg i.p. significantly ( $p < 0.001$ ) inhibited edema formation throughout the time course of the inflammatory reaction.

**In Vivo Interaction between Rosiglitazone, RU 486, and BADGE.** A reversion of the inhibitory effect of 3 mg/kg rosiglitazone was observed in animals pretreated with 10 mg/kg i.p. RU486 (Fig. 8), suggesting that the effect of rosiglitazone in vivo is caused by the interaction with glucocorticoid receptor. In fact, 10 mg/kg RU486 is able to reverse the inhibitory effect of 0.125 mg/kg i.p. dexamethasone, confirm-



**Fig. 5.** Effect of rosiglitazone, ciglitazone, dexamethasone, and RU486 on IL-6 production, IL-6 mRNA expression levels, and GR nuclear translocation in J774 cells. **A**, effect of 0.1 to 10  $\mu$ M rosiglitazone on IL-6 production in J774 macrophages. J774 cells were plated in DMEM (10% FCS) at  $2.5 \times 10^5$  cells/ml. Cells were stimulated with 100 ng/ml LPS for 24 h. Rosiglitazone was preincubated 2 h before LPS stimulation. Data shown are from three independent experiments and are expressed as mean  $\pm$  S.E.M. C, LPS-stimulated cells. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  versus C. **B**, J774 cells were plated in DMEM (10% FCS) at  $1.0 \times 10^6$  cells/ml. Cells were stimulated with 100 ng/ml LPS for 6 h. Rosiglitazone (Rosi; 10  $\mu$ M), 10  $\mu$ M ciglitazone (Cigli), and 1  $\mu$ M Dex were all preincubated 2 h before LPS stimulation. RU486 (RU; 400 nM) was added 2 h before rosiglitazone, ciglitazone, or dexamethasone treatment. Twenty-five cycles of PCR reaction of reverse-transcribed mRNA into cDNA were

ing the ability of RU486 to antagonize in vivo the interaction of dexamethasone with glucocorticoid receptor (Fig. 8, inset).

However, the inhibitory effect of rosiglitazone (3 mg/kg) was partially reversed also by the concomitantly administration of 10 mg/kg s.c. BADGE (Fig. 8), suggesting in vivo PPAR- $\gamma$  role in the anti-inflammatory effects of TZDs. It is interesting that when animals were pretreated with 10 mg/kg RU486 in combination with 10 mg/kg BADGE, the anti-inflammatory effect of rosiglitazone was completely reversed, suggesting that PPAR- $\gamma$  and steroid receptor signaling pathway could be interrelated in vivo (Fig. 8). Similar results were also observed during the late phase of the edema and with ciglitazone (data not shown). Treatment of rats with RU 486 and BADGE alone did not modify edema formation (data not shown).

**In Vivo Interaction between Actinomycin D and Rosiglitazone.** The inhibitory effect on edema formation by rosiglitazone is prevented in animals pretreated with 0.5 mg/kg i.p. actinomycin D, an inhibitor of RNA synthesis, suggesting that the mode of action of rosiglitazone involves the induction of the synthesis of "regulatory" proteins (Fig. 9).

**In Vivo Interaction between Dexamethasone and Rosiglitazone.** It is interesting that in animals treated with ineffective doses of both dexamethasone (0.06 mg/kg) and rosiglitazone (0.1 mg/kg), a synergic effect between these two drugs was observed throughout the time course of the carrageenin edema (Fig. 10).

## Discussion

Nuclear receptors are of major importance for intercellular signaling in animals because they converge different intra- and extracellular signals on the regulation of genetic programs. Such nuclear receptors are transcription factors that 1) respond directly through physical association with a variety of hormonal and metabolic signals, 2) integrate diverse signaling pathways because they correspond themselves to targets of post-translational modifications, and 3) regulate the activities of other major signaling cascades (Bourguet et al., 2000).

Both PPARs and GR are members of the nuclear hormone receptor superfamily (Willson et al., 2000) and they both play important roles in regulating several physiological and pathological processes such as metabolism, cell proliferation, inflammation, and immune responses (Newton, 2000). However, the results presented to date portray a somewhat conflicting story on the consequences of PPAR- $\gamma$  activation in inflammation and atherogenesis (Spiegelman, 1998). One

performed by using specific primers for IL-6 and  $\beta$ -actin as described under *Materials and Methods* (top). Densitometric analysis of mRNA expression of IL-6 normalized to expression levels of housekeeping gene  $\beta$ -actin (bottom). Data shown are from three independent experiments and are expressed as mean  $\pm$  S.E.M. N, unstimulated cells; C, LPS-stimulated cells. \*\*\*,  $p < 0.001$  versus N; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  versus C; + + +,  $p < 0.001$  versus Rosi or Dex without RU; + +,  $p < 0.01$  versus Cigli without RU. C, J774 cells were plated in DMEM (10% FCS) at  $1 \times 10^6$  cells/ml. Cells were stimulated with 10  $\mu$ M Rosi, 10  $\mu$ M Cigli, and 1  $\mu$ M Dex for 2 h. Thereafter, nuclear extracts were prepared as described under *Materials and Methods*. Western blot analysis for GR protein was performed as described under *Materials and Methods* (top); equal loading was confirmed by actin staining. Densitometric analysis of GR nuclear levels (bottom). Data shown are from three independent experiments and are expressed as mean  $\pm$  S.E.M. N, unstimulated cells. \*\*\*,  $p < 0.001$  versus N.

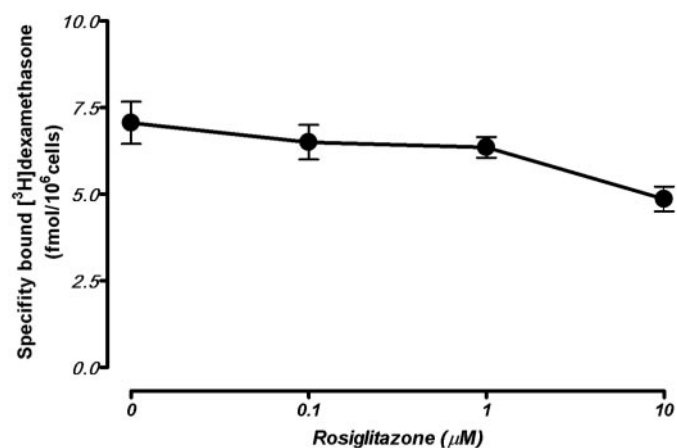


difficult in the explanation of the results presented to date is that many investigators have used the naturally occurring activator 15-deoxy- $\Delta^{12,14}$ -prostaglandin  $J_2$ , which also has cellular activity independent of PPAR- $\gamma$  (Rossi et al., 2000; Ianaro et al., 2003). Moreover, several studies, mainly conducted in vitro, have suggested a potential role of TZDs as anti-inflammatory agents (Daynes and Jones, 2002). Nevertheless, it has been demonstrated that TZDs exert anti-inflammatory effect only at a concentration higher than the  $K_d$  value for PPAR- $\gamma$  (Chawla et al., 2001; Oates et al., 2002). Furthermore, PPAR- $\gamma$  ligands were found to suppress the induction of COX-2 in PPAR- $\gamma$  (-/-) macrophages (Chawla et al., 2001), suggesting that this class of compounds could act via a PPAR- $\gamma$ -independent mechanism. Regardless, so far the possibility that certain PPAR- $\gamma$  ligands may have biological activities that are independent of PPAR- $\gamma$  has not been tested.

To clarify the role of PPAR- $\gamma$  ligands on inflammation, we decided to study their effect by using different cells lines such as E8.2 and E8.2/GR3 lacking or not GR, respectively, and both expressing PPAR- $\gamma$ , as well as murine macrophages J774 lacking PPAR- $\gamma$  and expressing GR.

TZDs showed no anti-inflammatory activity in cell line lacking GR. In contrast, in E8.2/GR3 expressing GR, TZDs exert anti-inflammatory activity, inhibiting both iNOS and IL-6 mRNA expression and consequently  $NO_2^-$  and IL-6 production by allowing GR nuclear translocation. It is interesting that TZDs induced GR nuclear translocation-exerting anti-inflammatory activity also in cells lacking PPAR- $\gamma$ . Furthermore, both in E8.2/GR3 and J774 cell lines the anti-inflammatory effect of TZDs was reversed by the GR antagonist RU486.

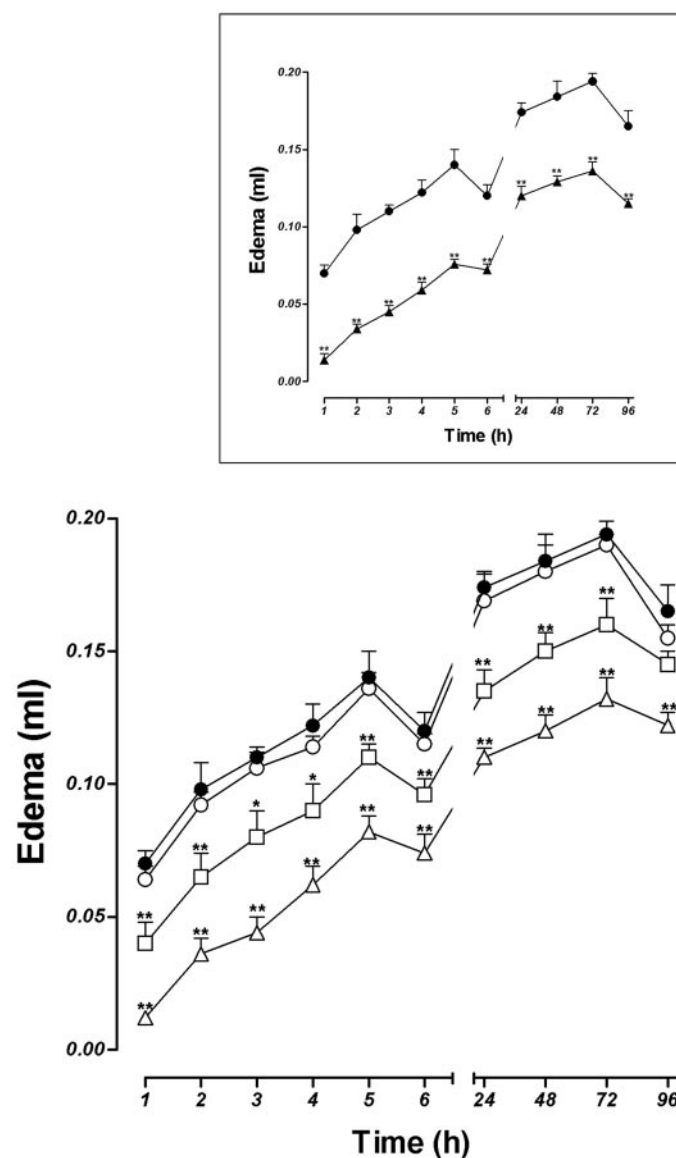
Moreover, our results obtained in vitro suggested that one of the possible mechanisms explaining the anti-inflammatory activity of TZDs could be their ability to activate GR nuclear translocation. Further experiments (e.g., knocking down GR in macrophages cell lines) will conclusively clarify this point. In addition, this study suggested that PPAR- $\gamma$  expression seems to be not required to TZDs-mediated GR nuclear translocation. PPAR- $\gamma$ -independent effects could be explained, at



**Fig. 6.** Effect of TZDs on dexamethasone-specific binding. J774 cells were exposed to the indicated concentrations of rosiglitazone for 1 h. Thereafter, the whole cell binding assay was performed with 10 nM [ $^3H$ ]dexamethasone for 2 h. Values are the mean  $\pm$  S.E.M. from three independent experiments.

least in part, by the interaction of TZDs with others PPARs (e.g., PPAR- $\delta$ ), as suggested by Welch et al. (2003).

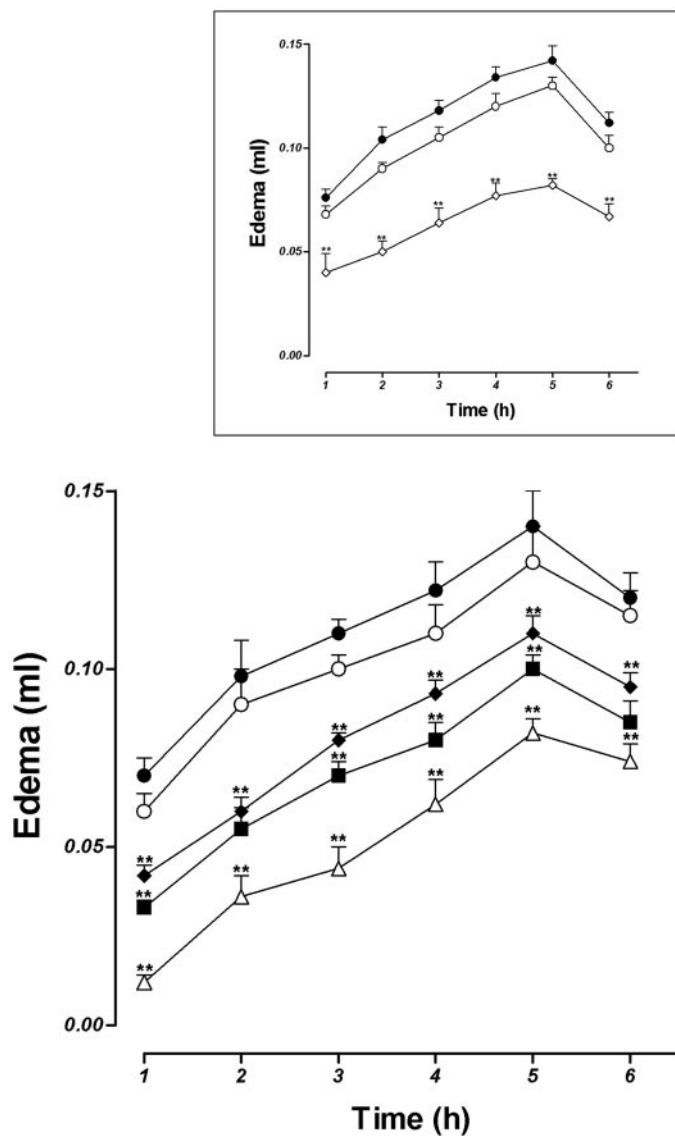
Moreover, by using a murine model of both acute and subchronic inflammation, the carrageenin-induced paw edema in mouse, we show that the anti-inflammatory activity exhibited by the PPAR- $\gamma$  agonists rosiglitazone and ciglitazone is reversed by the GR antagonist RU486 and only partially by PPAR- $\gamma$  antagonist BADGE, but it is necessary to administer both compounds to obtain a complete reversion of the anti-inflammatory effects of TZDs. In addition the inhibition by TZDs is prevented by actinomycin D, suggesting that their anti-inflammatory activity, as well as glucocorticoids, may involve the induction of the synthesis of regulatory proteins.



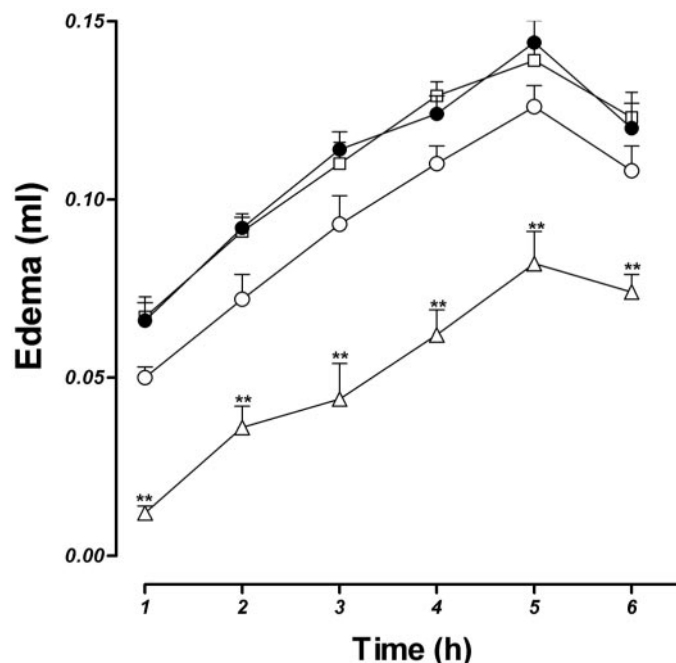
**Fig. 7.** Effect of rosiglitazone and ciglitazone on mouse carrageenin paw edema. Dose-effect of rosiglitazone (0.1 mg/kg, ○; 1 mg/kg, □; and 3 mg/kg, △) on paw edema induced by carrageenin. The edema induced by carrageenin alone (control group) is shown by ●. The results are expressed as mean  $\pm$  S.E.M., where  $n = 5$  to 8 animals. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ , versus control group. Inset, effect of 3 mg/kg ciglitazone (▲) on paw edema induced by carrageenin. The edema induced by carrageenin alone (control group) is shown by ●. The results are expressed as mean  $\pm$  S.E.M., where  $n = 5$  to 8 animals. \*\*,  $p < 0.01$  versus control group.

Steroid receptor antagonists have been invaluable tools in the dissection of the molecular mechanisms underlying steroid receptor activation of transcription (Fryer et al., 2000). It is possible to hypothesize that antagonistic action of RU486 is exerted at different steps of TZDs and glucocorticoids action. In fact, although our results *in vitro* clearly demonstrated that GR is required for the dexamethasone and TZD response, it might not be sufficient to explain TZDs' *in vivo* anti-inflammatory effects, as demonstrated by the activity of BADGE. BADGE is a synthetic ligand that binds to the receptor but is unable to transactivate genes through PPAR- $\gamma$ . However, BADGE can antagonize the ability of agonist ligands such as rosiglitazone to activate the transcrip-

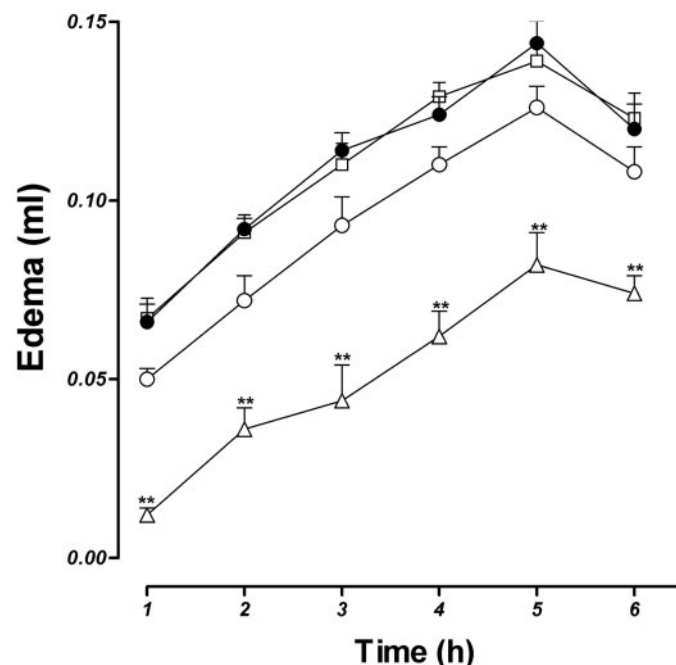
tional and adipogenic action of this receptor (Wright et al., 2000). Finally, our results could suggest *in vivo* the possibility of an interaction between GR and PPAR- $\gamma$  signaling pathways.



**Fig. 8.** In vivo interaction between rosiglitazone, RU486, and BADGE. Effect of 3 mg/kg rosiglitazone alone ( $\Delta$ ) or in combination with either 10 mg/kg RU486 ( $\blacklozenge$ ), 10 mg/kg BADGE ( $\blacksquare$ ), or both ( $\circ$ ) on paw edema induced by carrageenin. The edema induced by carrageenin alone (control group) is shown by  $\bullet$ . The results are expressed as mean  $\pm$  S.E.M., where  $n = 5$  to 8 animals. \*\*,  $p < 0.01$ , versus control group. Inset, effect of 0.125 mg/kg dexamethasone alone ( $\diamond$ ) or in combination with 10 mg/kg RU486 ( $\circ$ ) on paw edema induced by carrageenin. The edema induced by carrageenin alone (control group) is shown by  $\bullet$ . The results are expressed as mean  $\pm$  S.E.M., where  $n = 5$  to 8 animals. \*\*,  $p < 0.01$  versus control group.



**Fig. 9.** Effect of actinomycin D and rosiglitazone on mouse carrageenin paw edema. Effect of 3 mg/kg rosiglitazone alone ( $\Delta$ ), 0.5 mg/kg actinomycin D alone ( $\square$ ), or in combination ( $\circ$ ) on paw edema induced by carrageenin. The edema induced by carrageenin alone (control group) is shown by  $\bullet$ . The results are expressed as mean  $\pm$  S.E.M., where  $n = 5$  to 8 animals. \*\*,  $p < 0.01$  versus control group.



**Fig. 10.** In vivo interaction between dexamethasone and rosiglitazone. Effect of low doses of 0.1 mg/kg rosiglitazone alone ( $\Delta$ ), 0.06 mg/kg dexamethasone alone ( $\square$ ), or in combination ( $\blacklozenge$ ) on paw edema induced by carrageenin. The edema induced by carrageenin alone (control group) is shown by  $\bullet$ . The results are expressed as mean  $\pm$  S.E.M., where  $n = 5$  to 8 animals. \*\*,  $p < 0.01$  versus control group.



Some interesting data we obtained involves the anti-inflammatory activity exhibited by the combination of inactive doses of both dexamethasone and rosiglitazone, suggesting potential synergistic effects of these compounds on GR. We have demonstrated, in vitro, that TZDs did not significantly influence the binding of Dex to GR. These data are in agreement with the those of Cheron et al. (2004) showing that ciglitazone did not influence the binding of Dex to GR in RAW 264.7 macrophages. It would be straightforward to address how TZDs influence GR activity. One possible hypothesis is that TZDs act to up-regulate or activate factors that modulate GR activity. In fact, an alternative mechanism to explain TZDs' activity is the potentiation of GR transcription. This process is facilitated by molecules that interact with the DNA-bound GR and the transcription initiation complex among which steroid receptor coactivator 1 plays an important role (Feng et al., 1998). PPAR- $\gamma$  has also been reported to interfere with activator protein-1 and nuclear factor- $\kappa$ B activity in transient transfection assays, although it is not clear whether this transrepression mechanism is relevant in vivo. Ligands of PPAR- $\gamma$  stimulate the interaction between PPAR- $\gamma$  and the CREB-binding protein (CREB-binding protein/p300), which is an important coactivator for optimal activator protein-1-dependent transcription (Janknecht and Hunter, 1996). Hence, competition for limiting amounts of these proteins represents a mechanism for transrepression by nuclear receptors including PPAR- $\gamma$ . In fact, CREB-binding protein has recently been implicated in PPAR- $\gamma$ -dependent repression of both the iNOS and COX-2 genes (Li et al., 2000; Subbaramaiah et al., 2001). Moreover, TZDs' interaction with other PPARs can not be excluded. Further studies will address how TZDs can activate GR nuclear translocation and what consequences (if any) this activation may have at a transcriptional level.

Regardless, our results open new and exciting perspectives on the use of TZDs as anti-inflammatory agents, even if this study does not exclude PPAR- $\gamma$ -dependent action of TZDs on inflammatory processes linked to diseases in which they exert therapeutic effects (e.g., type 2 diabetes and atherosclerosis). How TZDs may affect the atherosclerotic process is an important issue because more than 1 million type II diabetics, who are already highly susceptible to atherosclerotic disease, are currently being treated with these compounds (Reginato and Lazar, 1999).

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