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Mechanism of the anti-inflammatory effect of thiazolidindiones. Relationship with the glucocorticoid pathway.

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Abbreviations: bisphenol A diglycidyl ether (BADGE); cyclooxygenase-2 (COX-2); dexamethasone

(DXM); glucocorticoids (GCs); glucocorticoid receptor (GR); inducible nitric oxide synthase

(iNOS); lipopolysaccharides (LPS); nuclear factor kappa-B (NF-κB); peroxisome proliferator-

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thiazolidindiones (TZDs).

Abstract

The glucocorticoid receptor (GR) and peroxisome proliferators-activated receptors (PPARs) play important roles in both physiological and pathological conditions such as cell differentiation, lipolisis, control of glucose metabolism, immunity and inflammation. In fact, recent studies suggest that the thiazolidindiones (TZDs) class of PPAR-γ 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-γ agonists rosiglitazone and ciglitazone is reverted by the GR antagonist RU486. 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 TZDs anti-inflammatory activity could be their ability to activate GR nuclear translocation. In addition, by using J774 cell line lacking PPAR-γ, we demonstrate that PPAR-γ expression could not be essential for TZDs-mediated GR nuclear translocation, thus explaining, at least in part, the molecular mechanism underlying their anti-inflammatory activity.

Introduction

Peroxisome proliferators-activated receptor-γ (PPAR-γ) is a member of the nuclear hormone receptor super family of ligand-activated transcription factors that are related to retinoid, steroid and thyroid hormone receptors (Evans, 1988). All members of this super family have a similar structural organization. A 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-α, PPAR-δ (also known as NUC1) and PPAR-γ (Lemberger et al., 1996). PPAR-γ 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 thiazolidindiones (TZDs) class of anti-diabetic drugs, which include rosiglitazone and ciglitazone and certain nonsteroidal anti-inflammatory drugs (Schoonjians et al., 1997; Willson et al., 2000). Recent studies suggest that the TZDs class of PPAR-γ 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-γ in regulating the inflammatory response, as 15d-PGJ$_2$ and other PPAR-γ agonists inhibit the expression of a variety of proteins with pro-inflammatory 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 Kd value for binding PPAR-γ or the concentration needed to elicit adipogenesis and insulin sensitization. Thus, a role for the receptor in mediating the anti-inflammatory activity of PPAR-γ ligands is not assured. Moreover, it has been recently shown that TZDs exert anti-inflammatory

effects in macrophages PPAR-γ (-/-) indicating that their anti-inflammatory activity is not only related to PPAR-γ (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 PPAR-γ, 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 (SRC1) 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-γ 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 due, at least in part, to their ability to activate GR nuclear translocation independently from PPAR-γ.

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 previously shown (Wei et al., 1998).

GR null mouse fibroblast cell E8.2 were maintained in 175 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Heidelberg, Germany) 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% fetal calf serum (FCS), 100 units/ml penicillin, 0.1 mg/ml streptomycin, 200 μg/ml of G418 (Geneticin, Gibco, Milan, Italy), 200 μg/ml of Hygromycin B (Gibco) and 1 μg/ml tetracycline (Sigma, Milan, Italy).

The murine monocyte/macrophage cell line J774 was from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom). 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^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 5 mM sodium pyruvate. All the cells were grown at 37°C in a humidified incubator under 5% CO_2.

RT-PCR of IL-6, iNOS and PPAR-γ

Total RNA was isolated from the cell using TRIzol (Invitrogen, Milan, Italy). Briefly, the cells were washed twice with ice-cold PBS, then 1 ml of TRIzol reagent was added to each 10 cm dish. The cells were collect after scraping, transferred to a microcentrifuge tube and homogenated by passing 5-10 times in 20 gage needle fitted onto 3 ml syringe.

200 μl of chloroform were added and the tube was shaken for 15 sec, followed by centrifugation at 12,000 x g 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 12,000 x g for 10 min. The supernatant was removed, and the RNA pellet was washed with 1 ml of 70% ethanol followed by centrifugation at 7,500 x g 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. 7 μg of total RNA was reverse-transcribed into cDNA by using oligo $(dT)_{12-18}$ primer (Invitrogen) and Superscript II Reverse Transcriptase (Invitrogen). One μg of cDNA was amplified by PCR using Taq Polymerase (Invitrogen) according to the manufacturer's instructions. The primers were: iNOS: sense: 5'TGGGAATGGAGACTGTCCCAG3', antisense: 5'GGGATCTGAATGTGATGTTTG3'; IL-6: sense 5'GGTCTTCTGGAGTACCATAGCTAC3',

antisense: 5'GGAATGTCCACAAACTGATATGC3'; PPAR-γ: sense 5'AAAGATGGAGTCCTCATCTC3', antisense: 5'AGCAGGGGGTGAAGGCTCAT3' (Nakamichi et al., 2003); β-ACTIN: sense 5' ATGAAGATCCTGACCGCGCGT3', antisense: 5'AACGCAGCTCAGTAACAGTCCG3'. The amplified fragments were 305bp, 327bp, 400bp and 584bp, respectively.

The PCR reaction was performed under the following conditions: a first cycle of denaturation at 94°C for 1 min 40 sec, then 25 or 30 cycles of denaturation at 94°C for 40 sec, annealing at 54°C (PPAR-γ) or 56°C (all others) for 40 sec, extension at 72°C for 1 min and 1 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 according to the manufacturer's instructions and the results expressed as ng/ml and represent the mean ± S.E.M. of *n* experiments runned in triplicate.

NO_2^- assay

The amount of NO_2^-, stable metabolites of nitric oxide, present in culture media from cells were measured 24 h after LPS from *E. Coli* (Fluka, Milan, Italy) plus or not IFN-γ stimulation with the Griess reaction as previously described (Ianaro et al., 2000). Results are expressed as nmol/ml and represent the mean ± S.E.M. of *n* experiments runned in triplicate.

Preparation of nuclear extracts

All the extraction procedures were performed on ice with ice-cold reagents. Stimulated or not E8.2/GR3 and J774 cells were washed twice with ice-cold PBS and centrifuged at 1500 x g 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 1500 x g at 4°C for 5 min, 1 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 13000 x g for 15 min, 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 µg) 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% non-fat dry milk in PBS-0.1% Triton X-100 for three hours 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, Santa Cruz, CA) goat antibody over night 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 (Dako, Milan, Italy) for 1 hour at room temperature. The immune complexes were visualized by the enhanced chemiluminescence method (Amersham, Cologno Monzese, Italy). Subsequently, the relative presence of GR and actin was quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

GR binding assay

The binding assay described by (Cheron et al., 2004) was used, with minor modifications. Briefly, J 774 cells ($1x10^6$/ml) were incubated in culture medium supplemented with 2.5% FCS and containing [^{3}H]dexamethasone (specific activity 88 Ci/mmol; Amersham, Milan, Italy) for 2 h at 37°C. TZDs treatment were performed 1 h prior dexamethasone. After incubation, monolayers were washed six times with 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 1-32 nM [³H]dexamethasone. For one point binding assays, 10 nM [3H]dexamethasone was used.

Animals

Male ICR mice (Harlan, Milan, Italy), weighing 25-30 g, were used in all experiments. Animals were provided with food and water *ad libitum*. The light cycle was automatically controlled (on 07 h 00min; off 19 h 00 min) and the room temperature thermostatically regulated to $22 \pm 1°C$. Prior to the experiments, animals were housed in these conditions for 3-4 days to become acclimatized. 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 into the rat right hind paw of 50 μl sterile saline containing 1% λ-carrageenin. Paw volumes were measured by a plethysmometer (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 (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 ± S.E.M. of *n* animals for *in vivo* experiments and of *n* experiments runned in triplicate for *in vitro* experiments. Comparisons were calculated by one-way

analysis of variance (ANOVA) 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 of 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 LPS 5 µg/ml and IFN-γ 100 U/ml for 24 h

caused a release of IL-6 (367.6 ± 17 pg/ml). Neither rosiglitazone (10 µM), ciglitazone (10 µM) nor

dexamethasone (1 µM) 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 as compared to E8.2 cells (940 ± 8.8 pg/ml). Surprisingly, rosiglitazone (10 µM),

ciglitazone (10µM) and dexamethasone (1 µM) all significantly (*p*<0.001) inhibited LPS/IFN-

induced IL-6 release by 60%, 52% and 73% respectively and, more interestingly, this inhibition was

significantly (*p*<0.001), albeit only partially, reverted by RU486 (400 nM) (Fig 1). In dose-response

experiments rosiglitazone (1 - 0.1 µM) 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, in order to verify that potential serum glucocorticoids do not cooperate for TZDs anti-

inflammatory effect (data not shown).

Stimulation of E8.2 or E8.2/GR3 cells with a combination of LPS 5 µg/ml and IFN-γ 100 U/ml for

6 h caused a significant increase of IL-6 mRNA expression levels compared to unstimulated cells

(Fig. 2A). Neither rosiglitazone (10 µM), nor ciglitazone (10 µM), nor dexamethasone (1 µM) all

pre-incubated 2 hours before LPS/IFN stimulation, modified LPS/IFN-induced IL-6 mRNA

expression in E8.2 cells (Fig. 2A). In contrast, rosiglitazone (10 µM), ciglitazone (10 µM) and

dexamethasone (1 μM) almost completely inhibited LPS/IFN-induced IL-6 mRNA expression levels in E8.2/GR3 and, more interestingly, this inhibition was, albeit only partially, reverted when the E8.2/GR3 were pre-challenged with RU486 (400 nM) 2 hours 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 β-actin, shown in Figure 2B, revealed a significant ($p<0.001$) inhibition of LPS/IFN-induced IL-6 mRNA expression in rosiglitazone (10 μM), ciglitazone (10 μM) and dexamethasone (1 μM) treated-cells by 68%, 60% and 88%, respectively (Fig. 2B). Pre-challenge with RU486 (400 nM) caused a partial but significant ($p<0.001$) reversion of this inhibition.

Effect of rosiglitazone, ciglitazone, dexamethasone and of RU486 on NO_2^- production and iNOS mRNA expression levels by E8.2 and E8.2/GR3 cells

The production of NO_2^- by unstimulated E8.2 and E8.2/GR3 cells was undetectable (<50 nmol/ml; n=4). Incubation of the cells with LPS/IFN for 24 h caused a substantial release of NO_2^- (23 ± 1.1 nmol/ml; n=9). Stimulation of E8.2 cells with LPS/IFN in presence of either rosiglitazone (10 μM) or ciglitazone (10 μM) or dexamethasone (1 μM) did not modify NO_2^- release (Fig. 1). Stimulation of E8.2/GR3 cells with LPS/IFN caused a substantial release of NO_2^- (29.5 ± 0.77 nmol/ml) as compared to unstimulated cells. As already shown for IL-6 release rosiglitazone (10 μM), ciglitazone (10 μM) and dexamethasone (1 μM), significantly ($p<0.001$) inhibited LPS/IFN induced NO_2^- release by 31%, 26% and 37% respectively (Fig. 1). When cells were stimulated with LPS/IFN in the presence of RU486 (400 nM), rosiglitazone (10 μM), ciglitazone (10 μM) and dexamethasone (1 μM) a reversion of the inhibitory effects exhibited by all drugs was observed (Fig. 1). In dose-response experiments rosiglitazone (1 μM) inhibited NO_2^- production by 17% ($p<0.05$), while rosiglitazone (0.1 μM) did not modified NO_2^- production compared to that observed in control cells (data not shown).

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Stimulation of E8.2 or by E8.2/GR3 cells with a combination of LPS 5 µg/ml and IFN-γ 100 U/ml for 6 h caused a significant increase of iNOS mRNA expression levels compared to unstimulated cells (Fig. 2A). Neither rosiglitazone (10 µM), nor ciglitazone (10 µM) nor dexamethasone (1 µM) all pre-incubated 2 hours before LPS/IFN stimulation, modified LPS/IFN-induced iNOS mRNA expression in E8.2 cells. In contrast, rosiglitazone (10 µM), ciglitazone (10 µM) and dexamethasone (1 µM) significantly ($p<0.001$) inhibited LPS/IFN-induced iNOS mRNA expression levels in E8.2/GR3 and, more interestingly, this inhibition was reverted when the E8.2/GR3 were pre-challenged with RU486 (400 nM) 2 hours 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 β-actin, shown in Figure 2B, revealed a significant ($p<0.001$) inhibition of LPS/IFN-induced iNOS mRNA expression in rosiglitazone (10 µM), ciglitazone (10 µM) and dexamethasone (1 µM), treated-cells by 28%, 22%, and 29%, respectively (Fig. 2B), whereas the pre-challenge with RU486 (400 nM) reverted 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-γ

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

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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 undetectable (<15 pg/ml; n=6). Incubation of J774 cells with LPS 0.1 µg/ml for 24 h caused a significant release of IL-6 (5900 ± 320 pg/ml). In dose-response experiments rosiglitazone (0.1-10 µM) inhibited IL-6 production by 2%, 15% ($p<0.01$) and 37% ($p<0.001$), respectively (Fig. 5A). Ciglitazone (10 µM) and dexamethasone (1 µM) 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, in order to verify that potential serum glucocorticoids do not cooperate for TZDs anti-inflammatory effect (data not shown).

Stimulation of J774 cells with LPS 0.1µg/ml for 6 h caused a significant increase of IL-6 mRNA expression levels compared to unstimulated cells (Fig. 5B). Rosiglitazone (10 µM), ciglitazone (10 µM) and dexamethasone (1 µM) all pre-incubated 2 hours 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. Interestingly, this inhibition was significantly ($p<0.001$) reverted when J774 were pre-challenged with RU486 (400 nM) 30 minutes before rosiglitazone, ciglitazone or dexamethasone treatment (Fig. 5B).

Effect of rosiglitazone, ciglitazone and dexamethasone on GR nuclear translocation in J774 cells

Interestingly, also in J774 cells TZDs and dexamethasone induced nuclear translocation of GR, as shown by western blot analysis (Fig. 5C), suggesting that PPAR-γ could be not essential for TZDs to exert anti-inflammatory effects.

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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 rosiglitazone (0.1-10 μM) did not significantly affect [^{3}H]dexamethasone-specific binding to GR (Fig. 6). Similar results were obtained with ciglitazone (data not shown).

Results *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 (0.1-1-3 mg/kg/i.p.), before and after carrageenin injection, reduced paw edema in a dose-dependent manner throughout the time course of the oedema. 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, while 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 Figure 7 insert, 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 RU486 (10 mg/kg/i.p.) (Fig. 8), suggesting that the effect of rosiglitazone *in vivo* is due to the interaction with glucocorticoid receptor. In fact, 10 mg/kg RU486 is able to revert the inhibitory effect of dexamethasone (0.125 mg/kg/i.p.) confirming the ability of RU486 to antagonize *in vivo* the interaction of dexamethasone with glucocorticoid receptor (Fig. 8 insert).

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-γ role in the anti-inflammatory effects of TZDs. Interestingly, when animals were pretreated with RU486 (10 mg/kg) in combination with BADGE (10 mg/kg) the anti-inflammatory effect of rosiglitazone was

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completely reverted, suggesting that PPAR-γ 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 actinomycin D (0.5 mg/kg/i.p.), 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

Interestingly, 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 (a) respond directly through physical association with a large variety of hormonal and metabolic signals; (b) integrate diverse signaling pathways because they correspond themselves to targets of post-translational modifications; and (c) regulate the activities of other major signaling cascades (Bourguet et al., 2000).

Both PPARs and GR are members of the nuclear hormone receptor super family (Willson et al., 2000) and they both play important role 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-γ activation in inflammation and atherogenesis (Spiegelman, 1998). One difficult in the explanation of the results presented to date is that many investigators have employed the naturally occurring activator 15dPGJ$_2$, which also has cellular activity independent of PPAR-γ

(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 Kd value for PPAR-γ (Chawla et al., 2001; Oates et al., 2002). Furthermore, PPAR-γ ligands were found to suppress the induction of COX-2 in PPAR-γ (-/-) macrophages (Chawla et al., 2001) suggesting that this class of compounds could act via a PPAR-γ-independent mechanism. Anyway, so far the possibility that certain PPAR-γ ligands may have biological activities that are independent of PPAR-γ have not been tested.

To clarify the role of PPAR-γ 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-γ, as well as murine macrophages J774 lacking PPAR-γ 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. Interestingly, TZDs induced GR nuclear translocation exerting anti-inflammatory activity also in cells lacking PPAR-γ. 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 TZDs anti-inflammatory activity 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-γ expression seems to be not required to TZDs-mediated GR nuclear translocation. PPAR-γ-indipendent effects could be at least partially explained by the interaction of TZDs with others PPARs (e.g. PPAR-δ), as suggested by Welch et al. (2003).

Moreover, by using a murine model of both acute and sub-chronic inflammation, the carrageenin-induced paw edema in mouse, we show that the anti-inflammatory activity exhibited by the PPAR-γ

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agonists rosiglitazone and ciglitazone is reversed by the GR antagonist RU486 and only partially by PPAR-γ antagonist BADGE, but it is necessary to administer both compounds in order 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.

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 TZDs 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-γ. However, BADGE can antagonize the ability of agonist ligands such as rosiglitazone to activate the transcriptional 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-γ signaling pathways.

An interesting data we obtained is 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 paper by 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 an important role is steroid receptor coactivator 1 (SRC1) plays an important role (Feng et al., 1998). PPAR-γ has also been reported to interfere with AP-1 and NF-κB activity

in transient transfection assays, although it is not clear if this transrepression mechanism is relevant *in vivo*. Ligands of PPAR-γ stimulate the interaction between PPAR-γ and the CREB-binding protein (CBP/p300) which is important co-activator for optimal AP-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-γ. In fact, CBP has recently been implicated in PPAR-γ-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.

Anyway, our results open new and exciting perspectives on the use of TZDs as anti-inflammatory agents, even if this study do not exclude PPAR-γ 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 one 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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Footnotes

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Legends to figures

Figure 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 x 10^5 cells/ml. Cells were stimulated with a combination of LPS 5 μg/ml and IFN-γ 100 U/ml for 24 h. Rosiglitazone (Rosi 10 μM), ciglitazone (Cigli 10 μM), dexamethasone (Dex 1 μM) were all pre-incubated 2 hours before LPS/IFN stimulation. RU486 (RU 400 nM) was added 2 hours before rosiglitazone or ciglitazone or dexamethasone treatment. (**A**) Effect of TZDs and dexamethasone on IL-6 production in E8.2 (black columns) and E8.2/GR3 (empty columns) cells. (**B**) Effect of TZDs and dexamethasone on nitrite production in E8.2 (black columns) and E8.2/GR3 (empty columns) cells. Data shown are from three independent experiments and are expressed as mean ± S.E.M. C= LPS/IFN stimulated cells. ***$p<0.001$ vs C; . °°°$p<0.001$ vs Rosi, Cigli or Dex without RU.

Figure 2. *Effect of rosiglitazone, ciglitazone and dexamethasone on IL-6 and iNOS mRNA expression in E82 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 x 10^6 cells/ml. Cells were stimulated with a combination of LPS 5 μg/ml and IFN-γ 100 U/ml for 6 h. Rosiglitazone (Rosi 10 μM), ciglitazone (Cigli 10 μM), dexamethasone (Dex 1 μM) were all pre-incubated 2 hours before LPS/IFN stimulation. RU486 (RU 400 nM) was added 2 hours before rosiglitazone or 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 β-actin as described under "Experimental Procedures". (**B**) densitometric analysis of mRNA expression of IL-6 (upper panel) and iNOS (lower panel) in both E8.2 (black columns) and E8.2/GR3 cells (empty columns) normalized to expression levels of housekeeping gene β-actin. Data shown are from three independent experiments and are expressed

as mean ± S.E.M. N=unstimulated cells; C= LPS/IFN stimulated cells. °°°$p<0.001$ vs N; ***$p<0.001$ vs C; +++$p<0.001$ vs Rosi, Cigli or Dex without RU.

Figure 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 1×10^6 cells/ml. Cells were stimulated with rosiglitazone (Rosi 10 µM), ciglitazone (Cigli 10 µM), and dexamethasone (Dex 1 µM) for 2 hours. Thereafter nuclear extracts were prepared as described under "Experimental Procedures".

Western blot analysis for GR protein was performed as described under "Experimental Procedures" (upper panel), equal loading was confirmed by actin staining. Densitometric analysis of GR nuclear levels (lower panel). Data shown are from three independent experiments and are expressed as mean ± S.E.M. N=unstimulated cells. ***$p<0.001$ vs N.

Figure 4. *RT-PCR of PPAR-γ*

(**A**) GR null mouse fibroblast E8.2 and GR reconstituted mouse fibroblast E8.2/GR3 cells were suspended in DMEM (10% FCS) at 1×10^6 cells/ml. Cells were stimulated with a combination of LPS 5 µg/ml and IFN-γ 100 U/ml (LPS/IFN) or rosiglitazone10 µM (Rosi) for 6 h. 25 cycles of PCR reaction of reverse-transcribed mRNA into cDNA were performed by using specific primers for PPAR-γ and β-actin as described under "Experimental Procedures". N=unstimulated cells. (**B**) Murine macrophages J774 were plated in DMEM (10% FCS) at 1×10^6 cells/ml. Cells were stimulated with LPS 100 ng/ml (LPS) or rosiglitazone 10 µM (Rosi) for 6 h. 30 cycles of PCR reaction of reverse-transcribed mRNA into cDNA were performed by using specific primers for PPAR-γ and β-actin as described under "Experimental Procedures". N=unstimulated cells.

Figure 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 rosiglitazone (0.1-10 μM) on IL-6 production in J774 macrophages. J774 cells were plated in DMEM (10 % FCS) at 2.5 x 10^5 cells/ml. Cells were stimulated with LPS 100 ng/ml for 24 h. Rosiglitazone was pre-incubated 2 hours before LPS stimulation. Data shown are from three independent experiments and are expressed as mean ± S.E.M. C= LPS stimulated cells. **$p<0.01$, ***$p<0.001$ vs C. (**B**) J774 cells were plated in DMEM (10% FCS) at 1.0 x 10^6 cells/ml. Cells were stimulated with LPS 100 ng/ml for 6 h. Rosiglitazone (Rosi 10 μM), ciglitazone (Cigli 10 μM), dexamethasone (Dex 1 μM) were all pre-incubated 2 hours before LPS stimulation. RU486 (RU 400 nM) was added 2 hours before rosiglitazone or ciglitazone or dexamethasone treatment. 25 cycles of PCR reaction of reverse-transcribed mRNA into cDNA were performed by using specific primers for IL-6 and β-actin as described under "Experimental Procedures" (upper panel). Densitometric analysis of mRNA expression of IL-6 normalized to expression levels of housekeeping gene β-actin. (lower panel) Data shown are from three independent experiments and are expressed as mean ± S.E.M. N=unstimulated cells; C= LPS stimulated cells. °°°$p<0.001$ vs N; **$p<0.01$, ***$p<0.001$ vs C; +++$p<0.001$ vs Rosi or Dex without RU, ++$p<0.01$ vs Cigli without RU. (**C**) J774 cells were plated in DMEM (10% FCS) at 1 x 10^6 cells/ml. Cells were stimulated with rosiglitazone (Rosi 10 μM), ciglitazone (Cigli 10 μM), and dexamethasone (Dex 1 μM) for 2 hours. Thereafter nuclear extracts were prepared as described under "Experimental Procedures". Western blot analysis for GR protein was performed as described under "Experimental Procedures" (upper panel); equal loading was confirmed by actin staining. Densitometric analysis of GR nuclear levels (lower panel). Data shown are from three independent experiments and are expressed as mean ± S.E.M. N=unstimulated cells. ***$p<0.001$ vs N.

Figure 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 [^{3}H]dexamethasone for 2 h. Values are the mean ± S.E.M. from three independent experiments.

Figure 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 solid circles (●). The results are expressed as mean ± S.E.M. where $n = 5-8$ animals. *$p< 0.05$; **$p< 0.01$, vs control group. *Insert*, effect of ciglitazone (3 mg/kg, ▲) on paw edema induced by carrageenin. The edema induced by carrageenin alone (control group) is shown by solid circles (●). The results are expressed as mean ± S.E.M. where $n = 5-8$ animals. **$p< 0.01$ vs control group.

Figure 8. *In vivo interaction between rosiglitazone, RU486 and BADGE*

Effect of rosiglitazone alone (3 mg/Kg, △) or in combination with either, RU486 (10 mg/kg, ◆) BADGE (10 mg/kg, ■) or both (○) on paw edema induced by carrageenin. The edema induced by carrageenin alone (control group) is shown by solid circles (●). The results are expressed as mean ± S.E.M. where $n = 5-8$ animals. **$p< 0.01$, vs control group. *Insert*, effect of dexamethasone alone (0.125 mg/kg, ◇), or in combination with RU486 (10 mg/kg, ○) on paw edema induced by carrageenin. The edema induced by carrageenin alone (control group) is shown by solid circles (●). The results are expressed as mean ± S.E.M. where $n = 5-8$ animals. **$p< 0.01$ vs control group.

Figure 9. *Effect of actinomycin D and rosiglitazone on mouse carrageenin paw edema*

Effect of rosiglitazone alone (3 mg/kg, △), actinomycin D alone (0.5 mg/kg, □) or in combination (○), on paw edema induced by carrageenin The edema induced by carrageenin alone (control group) is shown by solid circles (●). The results are expressed as mean ± S.E.M. where $n = 5-8$ animals. **$p<0.01$ vs control group.

Figure 10. *In vivo interaction between dexamethasone and rosiglitazone*

Effect of low doses of rosiglitazone alone (0.1 mg/kg, △), dexamethasone alone (0.06 mg/kg, □) or in combination (◆) on paw edema induced by carrageenin. The edema induced by carrageenin alone (control group) is shown by solid circles (●). The results are expressed as mean ± S.E.M. where $n = 5-8$ animals. **$p< 0.01$ vs control group.
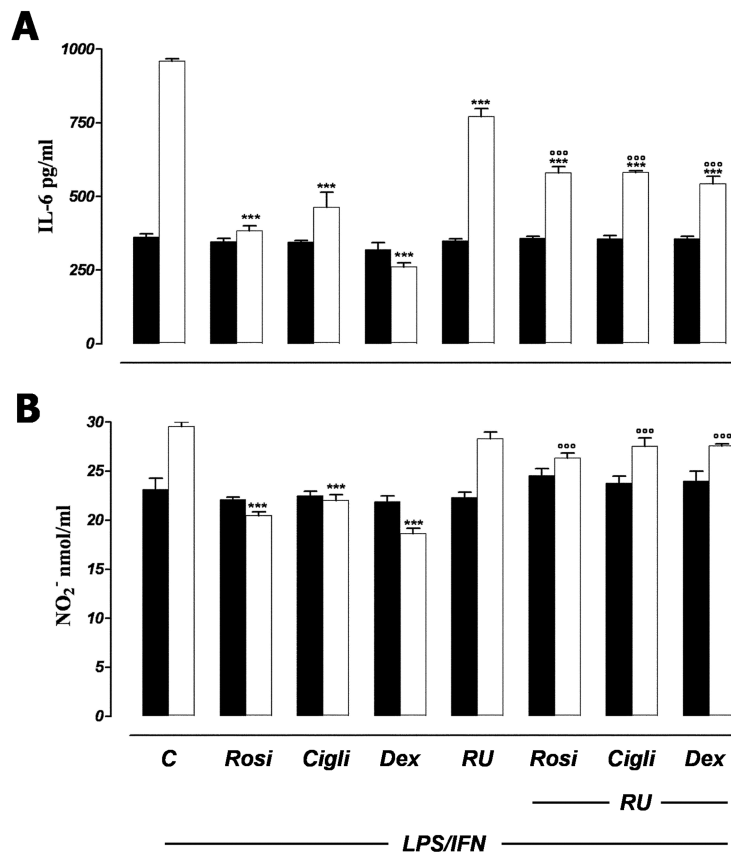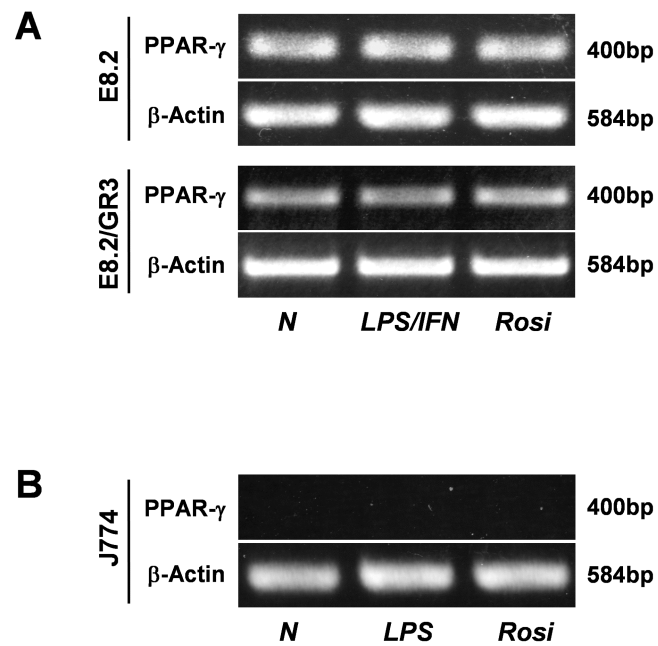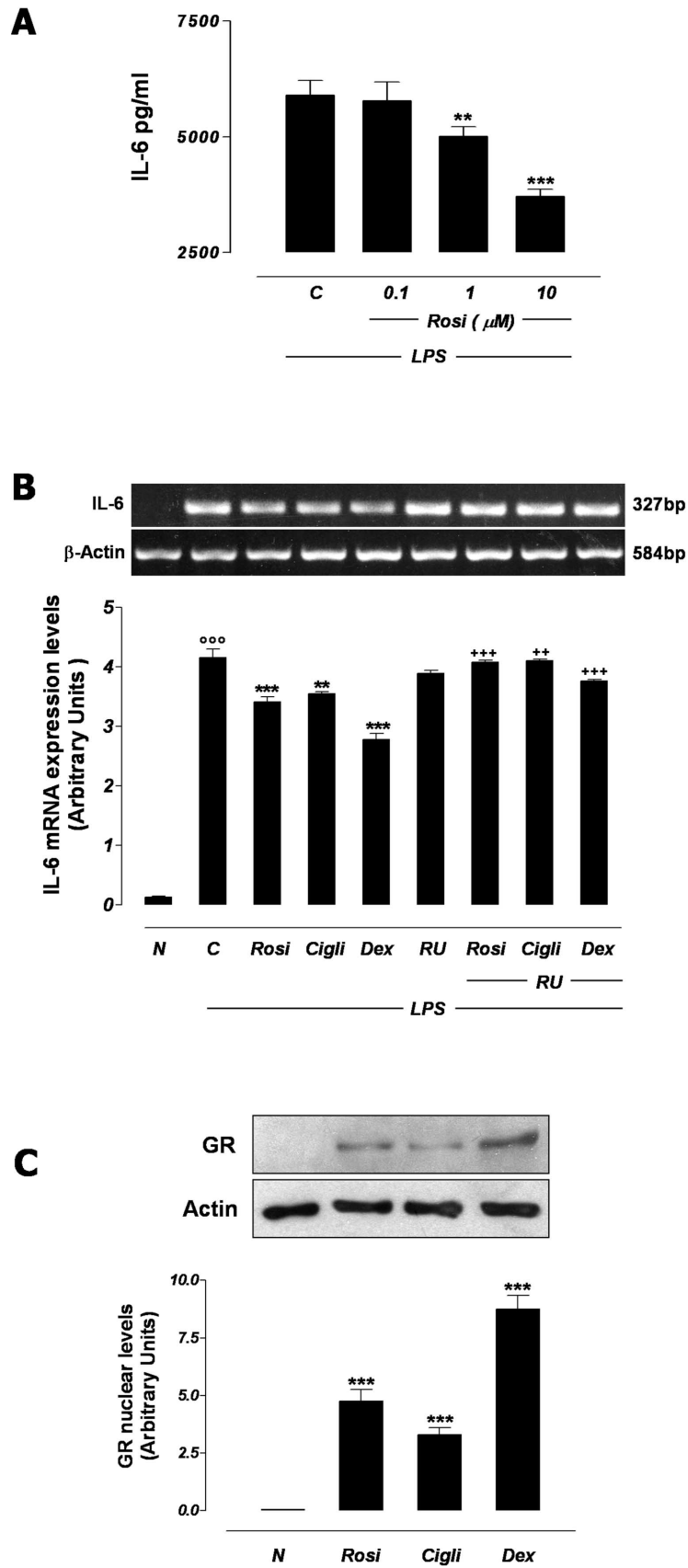
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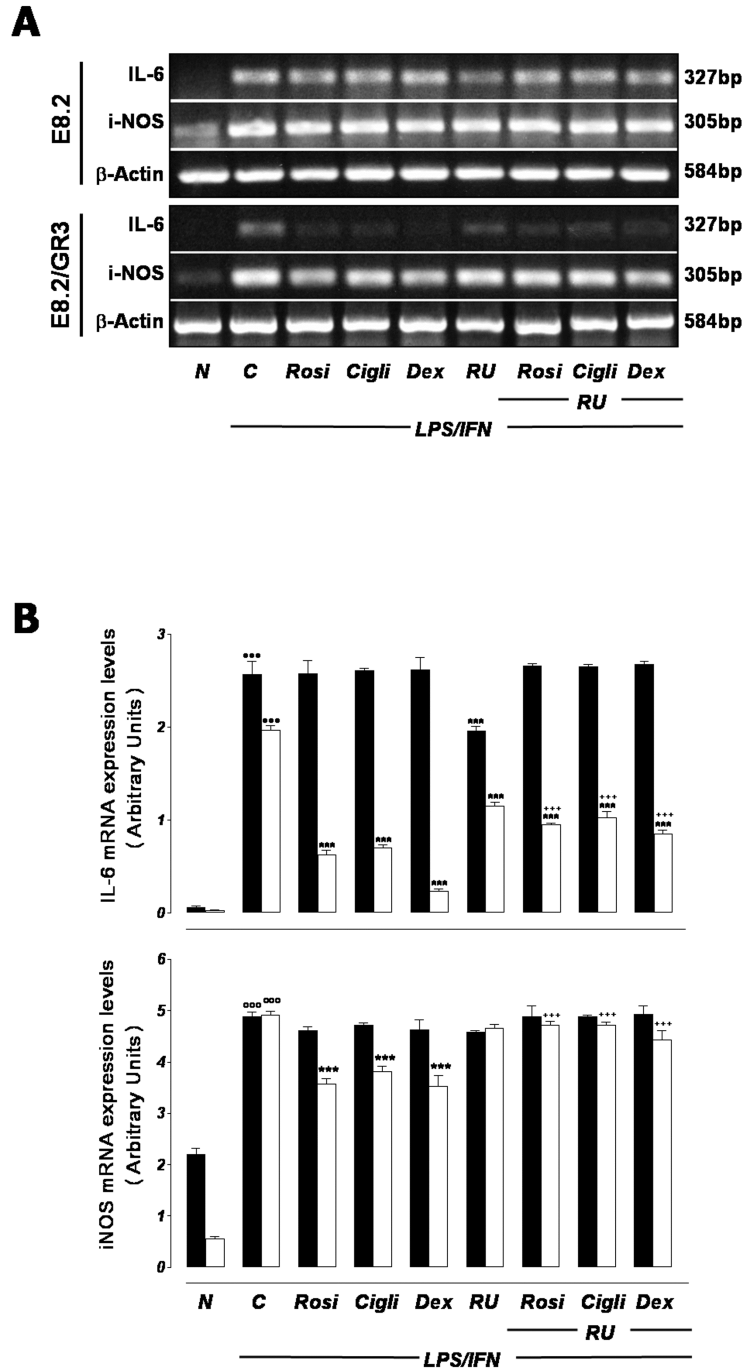
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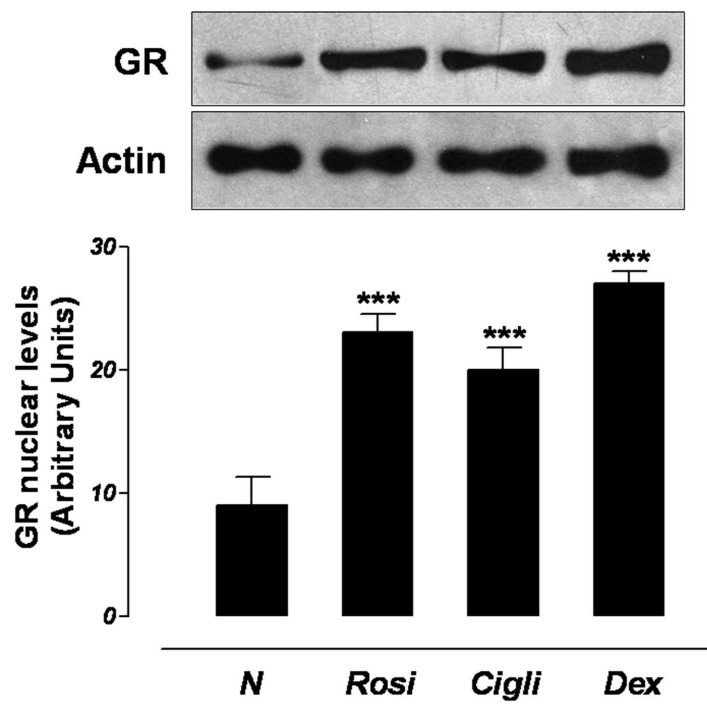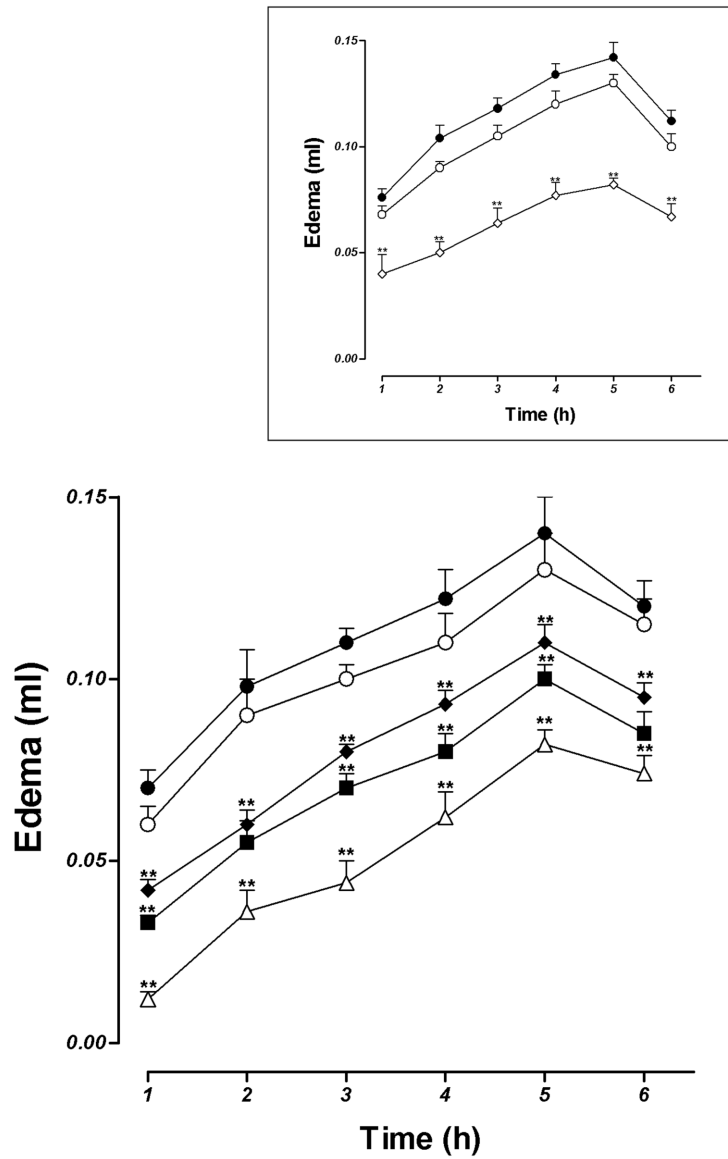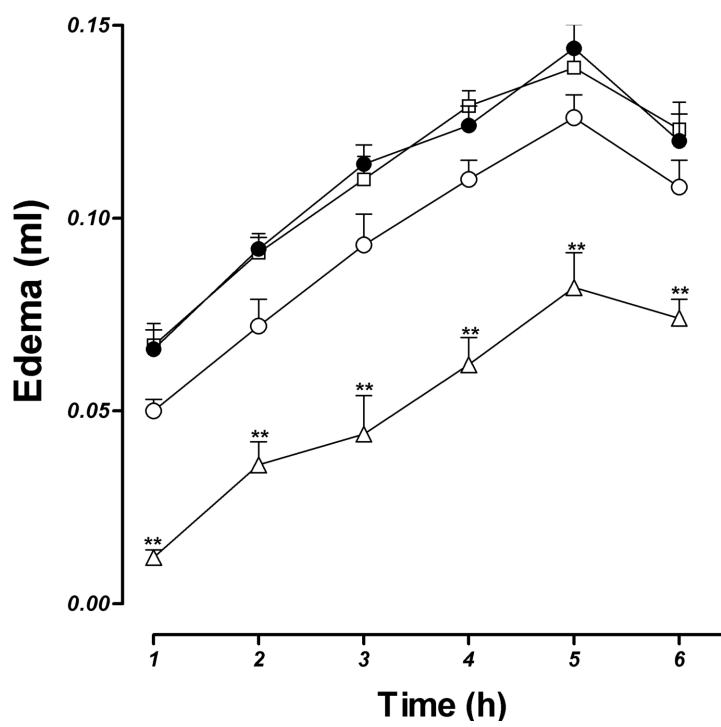
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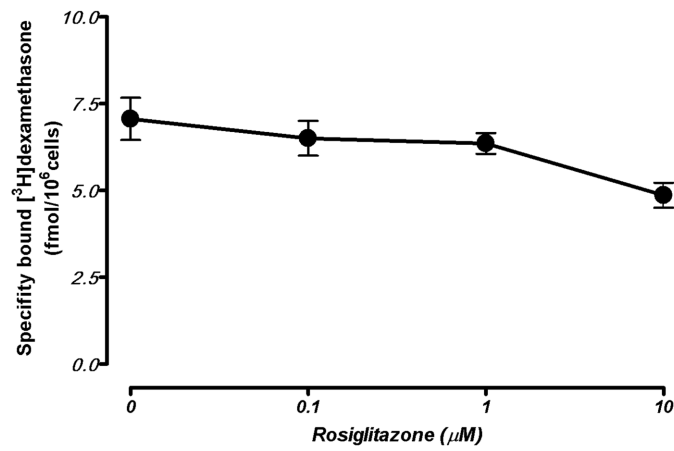
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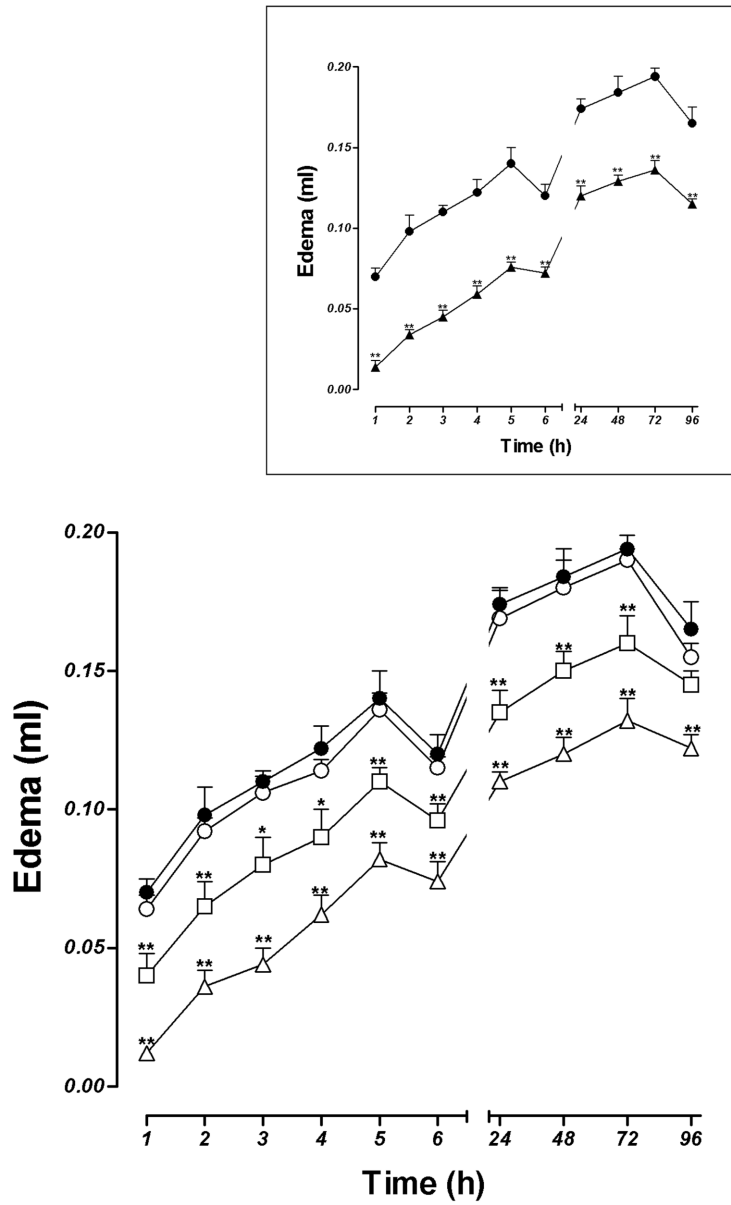
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