Rosiglitazone upregulates caveolin-1 expression in THP-1 cells through a PPAR-dependent mechanism

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Abstract Peroxisome proliferator-activated receptor γ (PPARγ) activation or overexpression induces caveolin-1 (cav-1) expression in several cell types. The objective of this study was to investigate if PPAR agonists could also regulate the cav-1 gene in macrophages and to explore the mechanisms involved. Our experiments demonstrated that rosiglitazone dose- and time-dependently increased cav-1 mRNA and protein in THP-1 macrophages. This induction was not observed in the presence of inhibitors of transcription or de novo protein synthesis. We also showed that the increase in cav-1 elicited by rosiglitazone was not related either to macrophage differentiation or to cellular apoptosis. The inductive effect seems to be dependent on PPAR activation, as the PPAR antagonist GW9662 abolished it. The activation of the liver X receptor with 22(*R*)-hydroxycholesterol also increased cav-1 mRNA, whereas the inactive (*S*) isomer did not. Finally, we identified a functional peroxisome proliferator response element in the cav-1 promoter that was activated upon rosiglitazone treatment in THP-1 macrophages.—Llaverias, G., M. Vázquez-Carrera, R. M. Sánchez, V. Noé, C. J. Ciudad, J. C. Laguna, and M. Alegret. **Rosiglitazone upregulates caveolin-1 expression in THP-1 cells through a PPAR-dependent mechanism. *J. Lipid Res.* 2004.** 45: **2015–2024.**

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Caveolins are a family of proteins that include three isoforms: caveolin-1 (cav-1) and cav-2 are expressed ubiquitously, most abundantly in endothelial cells, fibroblasts, adipocytes, and type I pneumocytes, whereas cav-3 is only expressed in striated muscle cells (1). Cav-1 is a 22 kDa integral membrane protein that has been identified as the principal marker for caveolae (2), plasma membrane specialized domains enriched in cholesterol and sphingolipids (3). Cav-1 has the ability to bind cholesterol (4), is essential for the assembly of caveolae (1), acts as a general inhibitor of several signaling molecules and enzymes, such as endothelial nitric oxide synthase (5), and is involved in vesicular transport and tumorigenesis (6).

Recently, it has been reported that ligands of peroxisome proliferator-activated receptor γ (PPARγ) upregulate cav-1 and cav-2 expression in human carcinoma cells (7). PPARs are ligand-activated transcription factors that heterodimerize with the 9-*cis*-retinoic acid receptor (RXR) and bind to specific peroxisome proliferator response elements (PPREs), modulating the transcription of genes involved in lipid metabolism, inflammation, and cell differentiation (8, 9). Synthetic ligands for PPARγ include the thiazolidinedione class of drugs, such as rosiglitazone, which are insulin-sensitizers used in the treatment of type 2 diabetes. Although PPARγ possesses some properties that are independent of DNA binding (10), thiazolidinediones exert many of their effects through PPARγ activation. Thus, activation of PPARγ leads to increased expression of the class B scavenger receptor CD36 in macrophages (11) and activates the liver X receptor (LXR) α, which in turn induces the expression of the ATP binding cassette transporter ABCA1 (12).

PPARγ is expressed in macrophages and in macrophage-derived foam cells in human atherosclerotic lesions. Cav-1 is also expressed in human macrophages and in some macrophage cell lines; specifically, cav-1 expression can be detected in THP-1 cells by immunoblotting and RT-PCR (13), whereas in J774 and RAW264.7 cells, cav-1 expression is detected by RT-PCR (14) but not by immunoblotting (13). The exact role of cav-1 in macrophages and in cellular cholesterol trafficking is not fully understood. Thus, some studies show that cav-1 expression is associated with an enhancement of cholesterol efflux (15, 16), whereas others indicate either no effect (17)

Abbreviations: cav-1, caveolin-1; EMSA, electrophoretic mobility shift analysis; LXR, liver X receptor; PPARγ, peroxisome proliferator-activated receptor γ; PPRE, peroxisome proliferator response element; RXR, 9-*cis*-retinoic acid receptor.

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or even a reduction (18). It has been suggested that cav-1 may play an indirect role by favoring cholesterol association with lipid rafts, from which it can move laterally to nonraft domains for efflux (1).

Because cav-1 expression is regulated by PPAR in other cell types, such as human adenocarcinoma (7) and hepatic cells (19), and both PPARγ and cav-1 are expressed in macrophages, the goal of the present study was to investigate the effect of the PPARγ agonist rosiglitazone on cav-1 expression in human THP-1 macrophages.

MATERIALS AND METHODS

Rosiglitazone was provided by GlaxoSmithKline, UK. Cell culture reagents were from Gibco, Invitrogen Corp. (Paisley, UK). Fetal bovine serum, PMA, actinomycin D, cycloheximide, geranylgeraniol, and 22(*R*)- and 22(*S*)-hydroxycholesterol were purchased from Sigma-Aldrich (St. Louis, MO). The PPARγ antagonist GW 9662 was from Cayman Chemical. Ultraspect was obtained from Biotecx (Houston, TX). RT-PCR reagents were from Invitrogen, except for the random hexamers and specific primers, obtained from Roche Diagnostics (Mannheim, Germany), and α-[^{32}P]dATP, from Amersham Biosciences (Freiburg, Germany). Rabbit polyclonal antibody against cav-1 was from BD Transduction Laboratories, and mouse monoclonal antibody against β-actin was from Sigma-Aldrich. Other general chemicals were obtained from commercial sources and were of analytical grade.

Cell culture

THP-1 cells were obtained from the European Collection of Cell Cultures and maintained in RPMI 1640 with 25 mM HEPES buffer (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37°C in 5% CO_2. Differentiation of monocytes to macrophages was achieved by exposing the cells to 50 nM PMA during 24 h (20). Rosiglitazone was added to cell culture dissolved in DMSO, and the final DMSO concentration was 0.1%. Cell viability was determined by measuring the ability of THP-1 cells to reduce 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (21).

RNA preparation and analysis

Total RNA was isolated using the Ultraspec reagent (Biotecx). Relative levels of specific mRNAs were assessed by RT-PCR. cDNA was synthesized from RNA samples by mixing 0.5 μg of total RNA and 125 ng of random hexamers as primers in the presence of 50 mM Tris-HCl buffer (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 200 units of Moloney murine leukemia virus reverse transcriptase, 20 units of RNAsin, and 0.5 mM of each deoxynucleoside 5′-triphosphate (dNTP; Sigma) in a total volume of 20 μl. Samples were incubated at 37°C for 60 min. A 5 μl aliquot of the RT reaction was then used for subsequent PCR amplification with specific primers. Each 50 μl PCR sample contained 5 μl of the RT reaction, 1.2 mM $MgCl_2$, 200 μM dNTPs, 0.25 μCi of [^{32}P]dATP (3,000 Ci/mmol; Amersham), 1 unit of Taq DNA polymerase, 0.5 μg of each primer, and 20 mM Tris-HCl, pH 8.5. To avoid unspecific annealing, cDNA and Taq polymerase were separated from primers and dNTPs using a layer of paraffin (reaction components contact only when paraffin fuses, at 60°C). The sequences of the sense and antisense primers used for amplification were as follows: cav-1, 5′-ACAAGCCCAACAACAAGGCCA-3′ and 5′-GAGGGCAGACAGCAAGCGGTA-3′; PPARγ, 5′-CATTCTGGCCCACCAACTTTGG-3′ and 5′-TGGAGATGCAGGCTCCACTTTG-3′; CD11b, 5′-AAGAGAACGCAAGGGGCTTCG-3′ and 5′-AGGGACAGGCCCAGGGACATG-3′; LXRα, 5′-AGCCCCCTTCAGAACCCACAG-3′ and 5′-AGGACACACTCCTCCCGCATG-3′; ABCA1, 5′-GGAGGCAATGGCACTGAGGAA-3′ and 5′-CCTGCCTTGTGGCTGGAGTGT-3′; and *gapdh*, 5′-CAGTCCATGCCATCACTGCCA-3′ and 5′-AGGTGGAGGAGTGGGTGTCGC-3′.

PCR was performed in an MJ Research Thermocycler equipped with a Peltier system and temperature probe. After an initial denaturation for 1 min at 94°C, PCR was performed for 22 (cav-1), 20 (PPARγ and CD11b), 23 (LXRα), and 18 (ABCA1) cycles. Each cycle consisted of denaturation at 92°C for 1 min, primer annealing at 60°C, and primer extension at 72°C for 1 min and 50 s. A final 5 min extension step at 72°C was performed. Five microliters of each PCR sample was electrophoresed on a 1 mm thick 5% polyacrylamide gel. The gels were dried and subjected to autoradiography using Kodak X-ray films to show the amplified DNA products. Amplification of each gene yielded a single band of the expected size (cav-1, 245 bp; PPARγ, 229 bp; CD11b, 195 bp; LXRα, 295 bp; ABCA1, 181 bp; and *gapdh*, 302 bp). Preliminary experiments were carried out with various amounts of cDNA and different numbers of cycles to determine nonsaturating conditions of PCR amplification for all of the genes studied. In these conditions, relative quantification of mRNA was assessed by the RT-PCR method (22). Radioactive bands were quantified by video densitometric scanning (Vilbert Lourmat Imaging). The expression of specific mRNAs is reported upon normalization using the *gadph* gene as an internal control.

Western blot analysis

Whole protein extracts (50 μg) from PMA-treated THP-1 cells were subjected to 12.5% SDS-PAGE. Proteins were then transferred to Immobilon polyvinylidene diflouride transfer membranes (Millipore, Bedford, MA). The membranes were blocked for 1 h at room temperature in TBS containing 0.5% Tween 20 and 5% nonfat dry milk, and immunological detection was performed using a rabbit polyclonal antibody raised against cav-1 (dilution, 1:5,000 in blocking buffer, 1 h at room temperature). Membranes were washed four times for 10 min in TBS plus 0.5% Tween 20 and 0.2% dry milk. Appropriate secondary antibody was diluted (1:2,000) and incubated with membranes as described for the primary antibody. Detection was achieved using the enhanced chemiluminescence detection system (Biological Industries). Blots were also incubated with a monoclonal antibody raised against β-actin (dilution, 1:5,000), used as a control of equal abundance of protein between the samples. The sizes of the detected proteins were estimated using protein molecular mass standards (Bio-Rad).

Analysis of apoptosis rate by flow cytometry and caspase-3 activity

Apoptosis was measured after 3, 6, 12, and 24 h of 1 μM rosiglitazone treatment as described previously (23). In brief, propidium iodide (10 μg/ml) and Triton X-100 (0.1%, v/v) were added to culture medium 30 min before cytofluorometry analysis and cells were collected from culture plates by pipetting. Flow cytometer experiments were carried out using an Epics XL flow cytometer (Coulter Corp., Hialeah, FL). The instrument was set up with the standard configuration: excitation of the sample was performed using as a standard a 488 nm air-cooled argon-ion laser at a power of 15 mW. Forward scatter, side scatter, and red (620 nm) fluorescence for propidium iodide were acquired. Optical alignment was based on the optimized signal from 10 nm fluorescent beads (Immunocheck, Epics Division).

For the determination of caspase-3 activity, we used the colorimetric substrate Ac-DEVD-*p*-nitroaniline (Oncogen) as described

by Jorda et al. (24). Twenty-four hours after treatment with 1 μM rosiglitazone, cells were collected in a lysis buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, and 0.1 mM EDTA, pH 7.4). Protein (50 μg/ml) was incubated with 200 M Ac-DEVD-p-nitroaniline in assay buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, and 0.1 mM EDTA, pH 7.4) on 96-well plates at 37°C for 24 h. Absorbance of the cleaved product was measured at 405 nm in a microplate reader (Bio-Rad). Results were expressed as absolute absorbance measured (mean ± SD of three experiments).

Electrophoretic mobility shift analysis

Nuclear extracts were prepared from THP-1 macrophages according to Dignam, Lebovitz, and Roeder (25) using 0.03% Triton for lysing the cells. Protein concentrations were determined by the Bradford method, and extracts were frozen in liquid N_2 and stored at −80°C. The cav-1-PPRE probe was generated by annealing the complementary oligonucleotides (Roche) from positions −278 to −258 of the human cav-1 gene promoter, relative to the translational start site. The sequences to construct the probe were as follows: upper strand, 5′-GCGCGGGACAACGTTCTCACT-3′; lower strand, 5′-AGTGAGAACGTTGTCCCGCGC-3′. The probe was end-labeled with T_4 polynucleotide kinase and γ-[^{32}P]ATP.

Electrophoretic mobility shift analysis (EMSA) was carried out basically as described by Noé et al. (26). The reaction mixtures (20 μl) contained 5% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 60 mM KCl, 2 μg of nuclear extract protein, 1 μg of herring sperm, 20,000 cpm of the DNA probe, and 25 mM Tris-HCl, pH 8.0. The mixture was incubated on ice for 15 min in the absence of the labeled probe and then for 30 min in its presence. Protein-DNA complexes were resolved by electrophoresis on a 5% polyacrylamide gel in 0.5× Tris/borate/EDTA at 4°C. The gels were dried and placed in contact with europium screens that were scanned by phosphorimaging.

Statistical analysis

Data are presented as means ± SD. An ANOVA, combined with the Student-Newman-Keuls test, was used to evaluate the statistical significance of the differences. The computer program GraphPad Instat was used for the calculations.

RESULTS

Rosiglitazone upregulates cav-1 mRNA levels

THP-1 monocytes were differentiated into macrophages by PMA exposure during 24 h and subsequently treated with various concentrations (5 nM to 2 μM) of rosiglitazone for 24 h. PCR analysis showed a marked upregulation of cav-1 mRNA, which was already significant at 0.05 μM rosiglitazone ($P < 0.01$). Maximal effect (3.4-fold induction) was attained at a dose of 1 μM (**Fig. 1A**). After addition of this dose of rosiglitazone, cav-1 mRNA levels started to increase at 3 h ($P < 0.05$ versus cells treated for 3 h with vehicle alone) and remained increased by 2- to 2.6-fold between 6 and 24 h (Fig. 1B).

Rosiglitazone induces cav-1 protein levels in PMA-treated THP-1 cells

To investigate whether changes in cav-1 mRNA levels were associated with modifications in the corresponding protein, Western blot analysis was performed. Our results show that rosiglitazone dose-dependently increased cav-1 protein levels (**Fig. 2A**), with maximal effects seen at 0.1–1 μM. Figure 2B shows the time-course effect of 1 μM rosiglitazone on cav-1 protein levels in THP-1 macrophages. After 6 h of treatment, cav-1 expression was significantly increased ($P < 0.05$), reaching maximal levels (3.3-fold increase) at 24 h of exposure.

Rosiglitazone does not induce the differentiation of THP-1 cells and has no effect on PPARγ mRNA levels

To determine if the upregulation of cav-1 by rosiglitazone is related to the ability of PPARγ ligands to promote cellular differentiation, we examined the time-course expression of the macrophage maturation marker CD11b. Rosiglitazone at 1 μM did not seem to induce the differentiation of THP-1 cells, as the mRNA levels of CD11b remain unaffected through a 24 h period (**Fig. 3A**). On the other hand, it has been reported that macrophage differ-

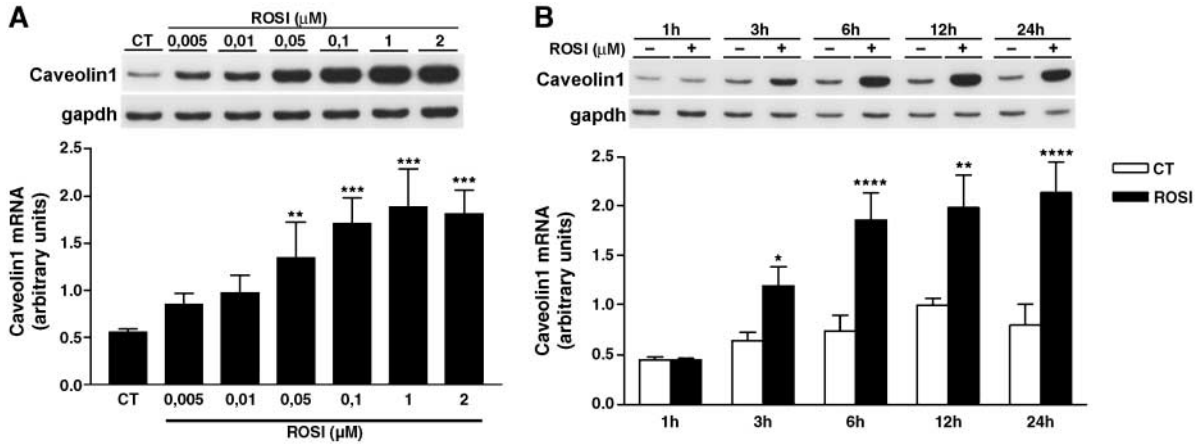

Fig. 1. Rosiglitazone upregulates caveolin-1 (cav-1) mRNA levels. A: Analysis of cav-1 mRNA levels in PMA-treated THP-1 cells incubated with various concentrations (0.005–2 μM) of rosiglitazone (ROSI) during 24 h. B: Analysis of cav-1 mRNA levels in PMA-treated THP-1 cells exposed to 1 μM rosiglitazone during 1, 3, 6, 12, and 24 h. Total RNA (0.5 μg) was analyzed by RT-PCR. Representative autoradiograms and quantifications of the gapdh-normalized mRNA levels are shown. Data are expressed as means ± SD of three independent experiments run in duplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ versus corresponding control (CT) cells.

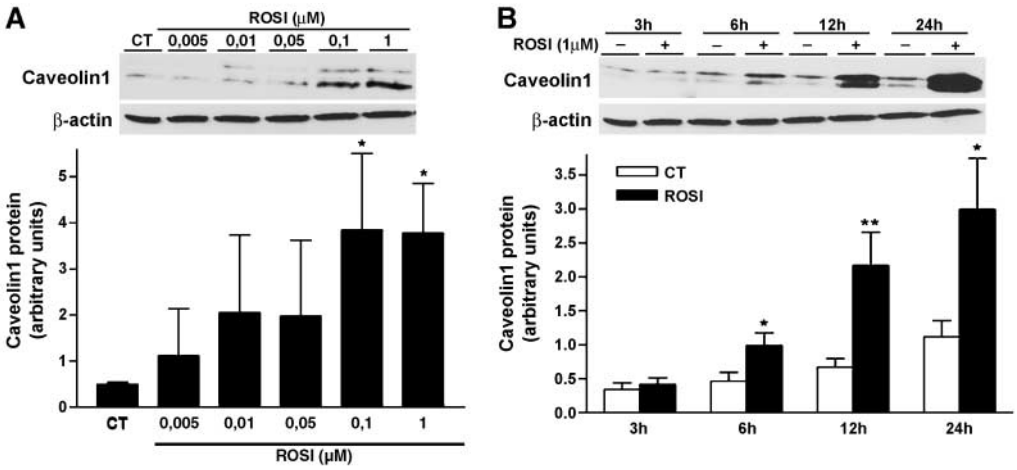

Fig. 2. Rosiglitazone induces cav-1 protein levels in THP-1 cells in a dose- and time-dependent manner. A: PMA-treated THP-1 cells were incubated with various concentrations (0.005–1 μM) of rosiglitazone (ROSI) during 24 h. B: PMA-treated THP-1 cells were incubated with 1 μM rosiglitazone during 3, 6, 12, and 24 h. Whole protein extracts (50 μg) were resolved on 12.5% SDS-polyacrylamide gel. The blots were analyzed with an anti-cav-1 antibody that detects two bands of ~22 kDa corresponding to the α and β isoforms. To show equal loading of protein, β-actin signal from the same blots are included. Representative autoradiograms and quantifications are shown. Data are expressed as means ± SD of three independent experiments. * $P < 0.05$ and ** $P < 0.01$ compared with corresponding control (CT) cells.

entiation is accompanied by an upregulation of PPARγ mRNA levels. Our results show that rosiglitazone treatment did not increase PPARγ gene expression over a 24 h period (Fig. 3B). Moreover, in contrast to rosiglitazone, when PMA-treated THP-1 cells were exposed to 50 nM PMA for a further 6 or 24 h, cav-1 mRNA levels were unaffected (Fig. 3C). These results strongly suggest that the increase in cav-1 mRNA levels induced by rosiglitazone is an early event that occurs independently of THP-1 differentiation.

Cav-1 induction by rosiglitazone is not related to cellular apoptosis

As other authors have suggested that an increase in cav-1 expression acts as a proapoptotic stimulus, we have investigated whether rosiglitazone treatment is associated with cellular apoptosis under the conditions of our study. The percentage of cells with subdiploid DNA content, assessed by flow cytometry, was not increased after treatment with 1 μM rosiglitazone (3–24 h), which indicates that the treatment does not induce the DNA fragmentation characteristic of apoptosis. Confirming these results, the activity of caspase-3, a key executor of apoptosis, was not increased in macrophages exposed to 1 μM rosiglitazone for 24 h (**Table 1**).

Upregulation of cav-1 is PPAR-dependent

To gain insight into the mechanism by which rosiglitazone upregulates cav-1 expression in THP-1 macrophages, we determined the effects of this compound in the presence or absence of inhibitors of gene transcription and protein synthesis. The increase in cav-1 mRNA levels induced by treatment with 1 μM rosiglitazone for 6 h was completely prevented by including the transcriptional inhibitor actinomycin D (1 μg/ml) or the inhibitor of protein synthesis cycloheximide (2 μg/ml) in the incubation medium (**Fig. 4A**). These results indicate that both transcriptional and posttranscriptional mechanisms may be involved in the action of rosiglitazone.

To determine whether the induction of cav-1 by rosiglitazone occurs through a receptor-dependent pathway, we examined the effect of the PPAR antagonist GW9662. This compound is a potent, selective antagonist that binds irreversibly to the PPARγ receptor (27). Addition of 20 μM GW9662 clearly blocked the increase in cav-1 mRNA levels induced in macrophages by treatment with 1 μM rosiglitazone for 6 h (Fig. 4B). As both PPARα and PPARγ are expressed in macrophages, and both are important regulators of genes involved in lipid metabolism in these cells, we tested the effect of a PPARα agonist, fenofibrate, on cav-1 expression. Our results show that fenofibrate (100 μM) also induced cav-1 mRNA levels by 1.8- and 2.5-fold after 6 and 24 h, respectively (Fig. 4C).

Coordinate induction of cav-1 and LXR mRNA by rosiglitazone and effect of LXR ligands and LXR antagonist on cav-1 mRNA levels

To explore whether an LXR-dependent pathway is involved in the upregulation of cav-1 by rosiglitazone, we first tested the effects of the potent LXR agonist 22(*R*)-hydroxycholesterol and of its inactive stereoisomer 22(*S*)-hydroxycholesterol. Consistent with previous reports, the mRNA levels of ABCA1, a well-known LXR target gene, are increased by 22(*R*)-hydroxycholesterol, whereas 22(*S*)-hydroxycholesterol, which does not activate LXR, was ineffective (**Fig. 5A**). Similarly, 22(*R*)-hydroxycholesterol also caused an increase in cav-1 mRNA levels at 24 h (2.6-fold; $P < 0.01$), whereas addition of the (*S*) isomer did not significantly induce cav-1 mRNA (Fig. 5A). On the other hand, the chronological pattern of the increase of cav-1 (Fig. 1B) and LXR (Fig. 5B) mRNA levels by 1 μM rosiglitazone was similar.

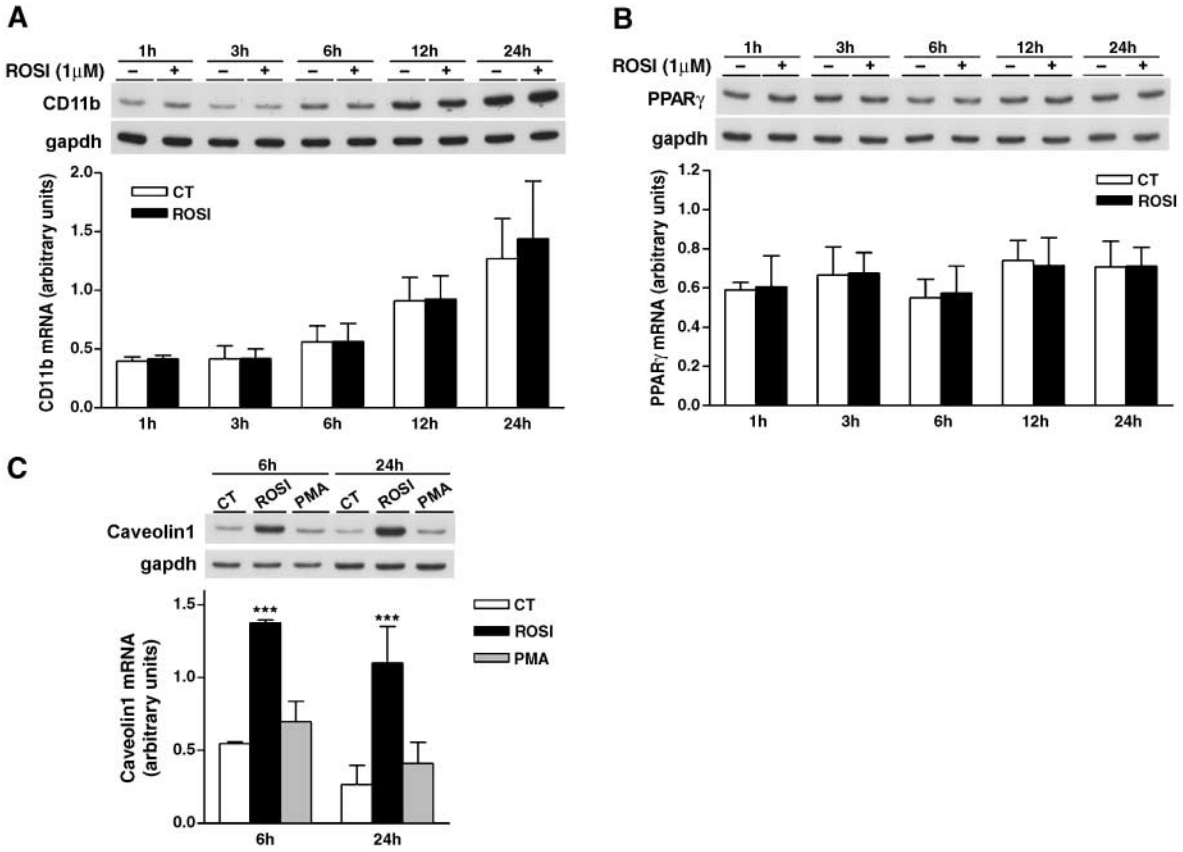

Fig. 3. Rosiglitazone does not induce differentiation of THP-1 cells and has no effect on peroxisome proliferator-activated receptor γ (PPARγ) mRNA levels. A and B: Analysis of CD11b (A) and PPARγ (B) mRNA levels in PMA-treated THP-1 cells exposed to 1 μM rosiglitazone (ROSI) during 1, 3, 6, 12, and 24 h. C: Analysis of cav-1 mRNA levels in PMA-treated THP-1 cells incubated with 1 μM rosiglitazone or 50 nM PMA for 6 and 24 h. Total RNA (0.5 μg) was analyzed by RT-PCR. Representative autoradiograms and quantifications of the gapdh-normalized mRNA levels are shown. Data are expressed as means ± SD of three independent experiments. *** $P < 0.001$ compared with control (CT) cells.

To further explore the involvement of the LXR pathway in the induction of cav-1 by rosiglitazone, experiments were performed using geranylgeraniol, which has been reported to act as an antagonist of LXR (28). In our conditions, geranylgeraniol significantly reduced by 24% ($P < 0.01$) the induction caused by 22(*R*)-hydroxycholesterol on ABCA1 mRNA (data not shown). Similarly, geranylgeraniol also inhibits rosiglitazone-induced cav-1 mRNA by 29%, as shown in Fig. 5C.

Identification of a PPRE in the cav-1 promoter

To determine if the effects of rosiglitazone could be attributable to the direct action of PPAR on the cav-1 gene, we analyzed the cav-1 promoter sequence (accession number AF019742) using the MATCH program version 1.0. This software is designed to search potential binding sites for transcription factor nucleotide sequences using the library of mononucleotide weight matrices from TRANSFAC 6.0. The search, performed using 0.75 and 0.70 as cutoffs for core and matrix similarity, respectively, revealed a putative PPAR/RXR binding site starting at position −281 relative to the translation start site (**Fig. 6A**). In contrast, no LXR binding site was found.

To check whether the putative PPRE was functional, we performed EMSA using as the probe a fragment of 21 bp containing the PPAR binding site sequence we found in the cav-1 promoter (cav-1-PPRE). Incubation of nuclear extracts from THP-1 cells with the cav-1-PPRE probe revealed the pattern of retardation shown in Fig. 6B. The intensity of the bands was increased by cellular treatment with 1 μM rosiglitazone in a time-dependent manner, with a maximum at 1 h.

DISCUSSION

In the present study, we show that rosiglitazone, at concentrations within the range that activate the PPARγ receptor (29), dose- and time-dependently induces the expression of cav-1 in THP-1 macrophages. The increase in cav-1 mRNA levels is already seen after 3 h of incubation with rosiglitazone and persists for 24 h. This rapid induction pattern is in accordance with the results obtained by Burgermeister, Tencer, and Liscocitch (7) in human carcinoma cells, in which several PPARγ ligands induced an acute upregulation of cav-1 mRNA levels. In the liver, overexpression of PPARγ also induces cav-1 expression, together with other genes that are expressed in adipo-

TABLE 1. Rosiglitazone does not increase the percentage of subdiploid cells or caspase-3 activity in THP-1 macrophages

Time	Control	Rosiglitazone (1 μM)
Percentage of subdiploid cells		
3 h	1.59 ± 0.37	1.46 ± 0.36
6 h	1.58 ± 0.11	1.86 ± 0.71
12 h	2.48 ± 0.26	2.62 ± 0.38
24 h	1.80 ± 0.16	2.04 ± 0.44
Caspase-3 activity		
24 h	0.106 ± 0.003	0.121 ± 0.025

Analysis of apoptosis rate by flow cytometry in PMA-treated THP-1 cells exposed to 1 μM rosiglitazone during 3, 6, 12, and 24 h. Propidium iodide (10 μg/ml) and Triton X-100 were added to culture medium 30 min before cytometric analysis. Cells were then collected from culture plates by pipetting, and cytofluorometry analysis was carried out using an Epics XL flow cytometer. Data are expressed as means of the percentage of positive propidium iodide cells ± SD of three independent experiments run in duplicate. Determination of caspase-3 activity in PMA-treated THP-1 cells exposed to 1 μM rosiglitazone during 24 h is also shown. Cells were collected in lysis buffer, and protein (50 μg) was incubated with 200 M Ac-DEVD-*p*-nitroaniline in assay buffer at 37°C for 24 h. Absorbance of the cleaved product was measured at 405 nm. Results are expressed as means of absorbances ± SD of three independent experiments.

cytes, such as adiponectin, aP2, and CD36 (19). Interestingly, these genes are also expressed in macrophages under the control of PPARγ. In fact, macrophages and adipocytes share several features: their differentiation is promoted by PPARγ activation, both cell types can accumulate large amounts of lipids, and both cell types express several common genes specifically involved in the control of lipid homeostasis.

Cav-1 has been suggested to act as a growth-inhibitory protein in oncogenically transformed cells, and this effect has been attributed in part to its ability to promote cell differentiation (6). However, in breast and colon cancer cells, the induction of caveolin expression by PPARγ ligands is independent of cell differentiation (7). On the other hand, ligand activation of the PPARγ/RXR heterodimer has been shown to promote the differentiation of monocytic cells (11), and cav-1 expression is induced in THP-1 cells upon differentiation (13). Therefore, we wanted to test whether the increase in cav-1 expression induced by rosiglitazone in our cells was a direct consequence of a differentiation-promoting effect of the PPARγ ligand. Our results show that the mRNA levels of CD11b, a widely used marker of macrophage differentiation (30), are not increased in cells treated with rosiglitazone for 3–24 h, whereas cav-1 expression is already induced at 3 h of rosiglitazone exposure. Therefore, the increase in cav-1 seems to occur without further cell differentiation. On the other hand, we detected no increase in PPARγ mRNA levels after rosiglitazone treatment. As it has been shown that differentiation of THP-1 cells upregulates PPARγ (31), these results suggests that, under the conditions of our assay, the addition of rosiglitazone does not promote further macrophage maturation. Finally, treatment of the cells with PMA for another 24 h, which is thought to increase cellular differentiation, does not cause an induction of cav-1 mRNA levels. Taking these results together, we propose that the induction of cav-1 found in our study seems to be independent of cellular differentiation, similar to what Burgermeister, Tencer, and Liscocitch (7) described in cancer cells.

In a recent publication, it has been suggested that the increase in cav-1 expression in macrophages treated with 10 μM simvastatin is associated with cellular apoptosis (32). We did not observe any change in the morphology of the cells indicative of apoptosis in any of the conditions

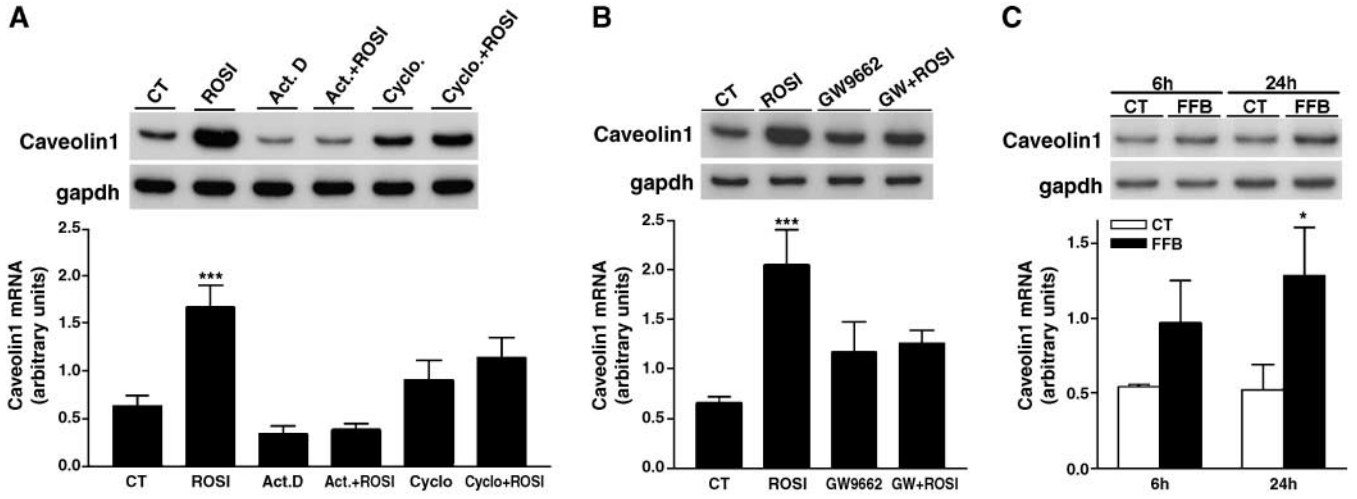

Fig. 4. Upregulation of cav-1 is PPAR-dependent. A: Analysis of cav-1 mRNA levels in PMA-treated THP-1 cells incubated with actinomycin D (Act. D; 1 μg/ml) or cycloheximide (Cyclo; 2 μg/ml) with or without 1 μM rosiglitazone (ROSI) during 6 h. B: Analysis of cav-1 mRNA levels in PMA-treated THP-1 cells incubated with 1 μM rosiglitazone, 20 μM GW 9662, or both drugs (the PPARγ antagonist was added 0.5 h before rosiglitazone) during 6 h. C: Analysis of cav-1 mRNA levels in PMA-treated THP-1 cells exposed to 100 μM fenofibrate for 6 and 24 h. Total RNA (0.5 μg) was analyzed by RT-PCR. Representative autoradiograms and quantifications of the gapdh-normalized mRNA levels are shown. Data are expressed as means ± SD of three independent experiments run in duplicate. * $P < 0.05$ and *** $P < 0.001$ versus corresponding control (CT) cells.

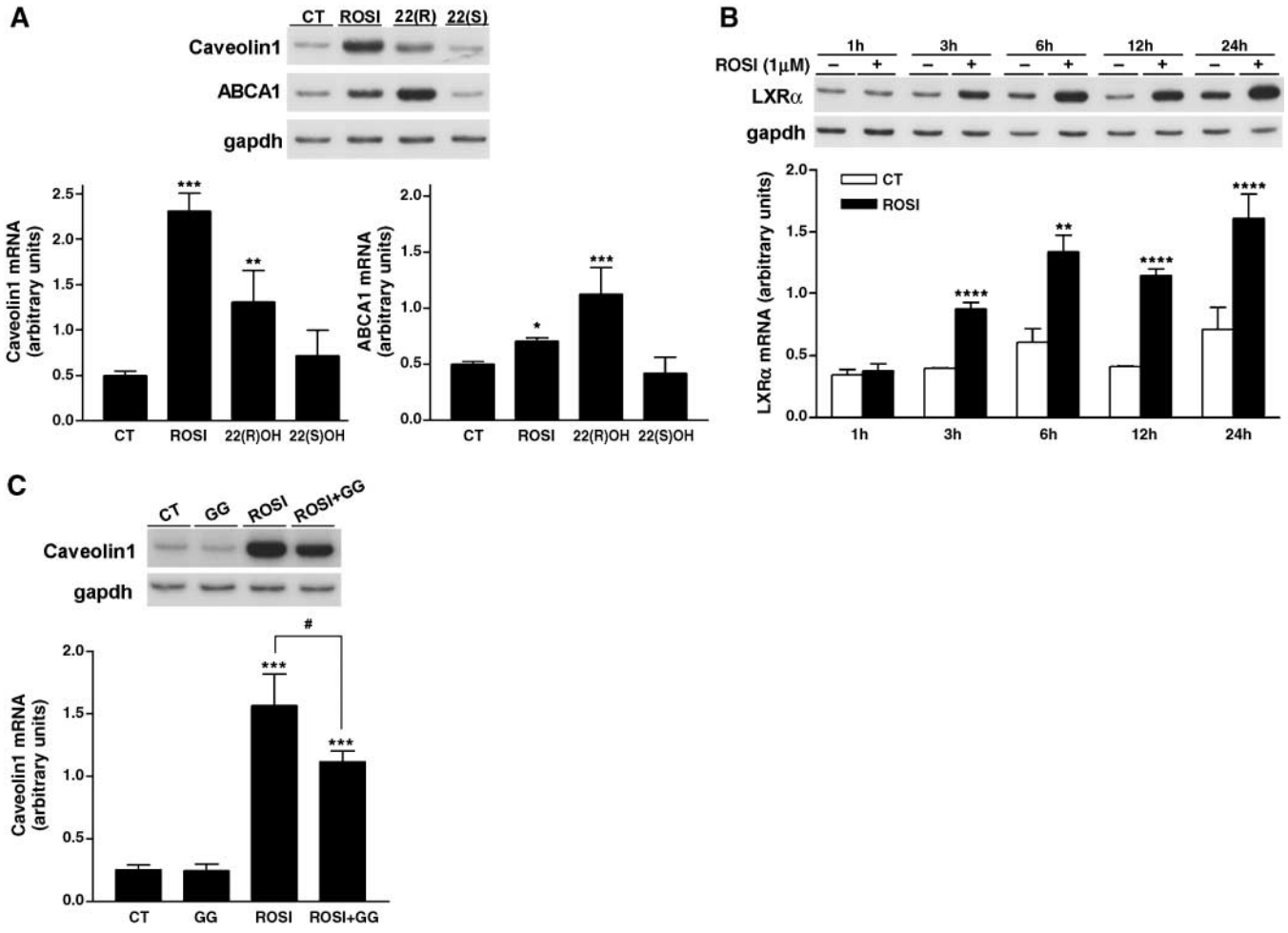

Fig. 5. Coordinate induction of cav-1 and liver X receptor (LXR) mRNA by rosiglitazone. Effect of LXR ligands and LXR antagonist on cav-1 mRNA levels. A: Analysis of ABCA1 and cav-1 mRNA levels in PMA-treated THP-1 cells incubated with 1 μM rosiglitazone (ROSI) and 10 μM 22(*R*)- or 22(*S*)-hydroxycholesterol during 24 h. B: Analysis of LXRα mRNA levels in PMA-treated THP-1 cells exposed to 1 μM rosiglitazone during 1, 3, 6, 12, and 24 h. C: Analysis of cav-1 mRNA levels in PMA-treated THP-1 cells incubated with 1 μM rosiglitazone, 50 μM geranylgeraniol (GG), or both drugs (the LXRα antagonist was added 0.5 h before rosiglitazone) during 24 h. Total RNA (0.5 μg) was analyzed by RT-PCR. Representative autoradiograms and quantifications of the gapdh-normalized mRNA levels are shown. Data are expressed as means ± SD of three independent experiments run in duplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ versus corresponding control (CT) cells; # $P < 0.01$ versus rosiglitazone-treated cells.

tested. Moreover, the number of cells with subdiploid DNA content and caspase-3 activity was not increased after treatment with 1 μM rosiglitazone, indicating that, in our case, the induction in cav-1 expression is not accompanied by apoptosis of the cells. The differences in the results obtained by Gargalovic and Dory (32) may be attributed to the use of different cell models, as it has been reported that the proapoptotic effects of caveolin are cell-specific. In addition, the increase in cav-1 induced by simvastatin in this study was ~20-fold, whereas our results show maximal increases in cav-1 expression of ~3-fold.

To test whether transcriptional regulation is involved in the induction of cav-1 by rosiglitazone, actinomycin D and cycloheximide were used to inhibit RNA and de novo protein synthesis, respectively. Our results show that rosiglitazone does not alter cav-1 transcript levels in the presence of actinomycin D, indicating that the PPARγ agonist affects gene transcription. Inhibition of de novo protein synthesis with cycloheximide also abolishes the ability of rosiglitazone to increase the expression of the cav-1 gene. Similarly, Lopez-Solache et al. (33) showed that blockage of protein synthesis with cycloheximide prevents the thiazolidinedione-induced increase of UCP2 mRNA in L6 myotubes, suggesting an indirect stimulus on UCP-2 transcription. Rosiglitazone could also indirectly affect cav-1 transcription by a mechanism involving the PPARγ-dependent induction of a protein that stimulates the gene. In contrast, other effects of glitazones are independent of PPARγ activation, as shown by the failure of actinomycin D and cycloheximide to counteract them (34, 35).

Supporting the involvement of PPAR activation, the increase in cav-1 expression induced by rosiglitazone is suppressed in the presence of the PPARγ antagonist GW9662. However, the inductive effect is not PPARγ-specific, as the PPARα agonist fenofibrate also increases cav-1 mRNA levels. At the concentration used in this study (100 μM), feno-

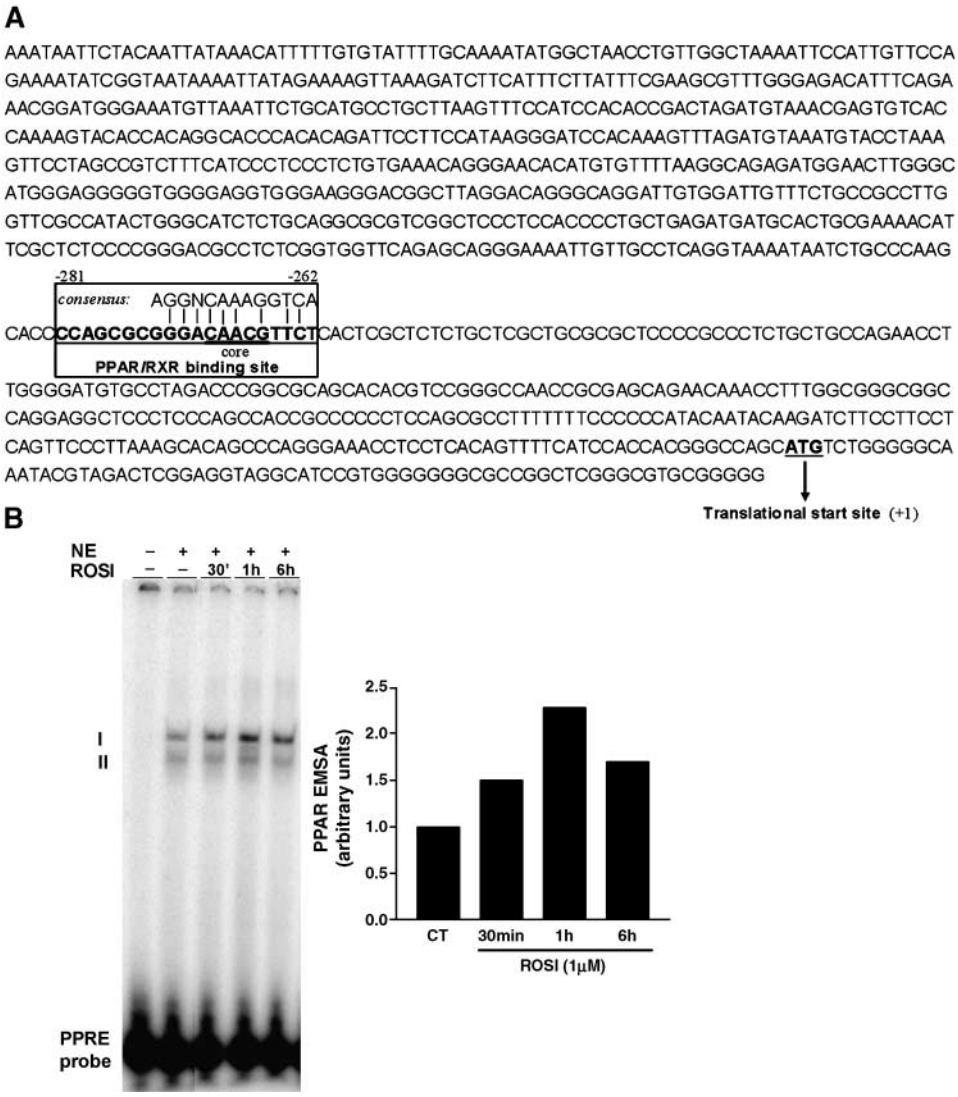

Fig. 6. The cav-1 gene promoter sequence contains a putative PPAR/9-*cis*-retinoic acid receptor (RXR) binding site. A: Sequence corresponding to the human cav-1 5′ flank. The putative PPAR/RXR binding site is located between −281 and −262 bp relative to the translational start site. B: Phosphorimaging analysis of EMSA using the ^{32}P-labeled cav-1-peroxisome proliferator response element (PPRE) and nuclear extract (NE) from THP-1 cells incubated with 1 μM rosiglitazone (ROSI) for the indicated periods of time. The quantification of the two bands is shown at right. CT, control.

fibrate only activates PPARα, as it has been reported that the IC_{50} for PPARγ activation in human tissues is 300 μM (36).

Burgermeister, Tencer, and Liscocitch (7) suggested that rosiglitazone may induce caveolin expression by indirect mechanisms, such as interaction with other transcription factors. Although it is not known which transcription factors mediate caveolin upregulation, it was recently suggested that nuclear receptors can be involved (37, 38). LXRs are members of the nuclear receptor superfamily that act as intracellular cholesterol sensors (39). Activation of PPARγ in macrophages induces LXRα expression and, as a consequence, upregulates proteins that play a key role in cholesterol efflux, such as apolipoprotein E and ABCA1 (40, 41). Our results show that treatment of macrophages with the naturally occurring LXR ligand 22(*R*)-hydroxycholesterol increases cav-1, as well as ABCA1, mRNA levels. Importantly, treatment with the synthetic (*S*) stereoisomer, which does not function as a LXR agonist in cells (42), induces neither ABCA1 nor cav-1 gene expression. These results could indicate the existence of a PPARγ-LXRα-cav-1 regulatory pathway, similar to that observed for other genes related to cholesterol efflux, such as ABCA1 and apolipoprotein E.

However, our experiments using an LXR antagonist show that the increase in cav-1 mRNA induced by rosiglitazone is reduced by 30%, whereas the use of a PPARγ antagonist completely abolishes this effect. In addition, the PPAR agonist has a greater effect on cav-1 expression compared with a specific LXR agonist, whereas the opposite occurs with ABCA1 mRNA. These results suggest that the induction of cav-1 expression by rosiglitazone is primarily a PPAR response.

To gain further insight into the mechanism regulating cav-1 expression, we searched for PPAR or LXR binding sites in the human cav-1 promoter. We identified, by computational means, a putative binding site for PPAR/RXR, not previously reported, between −281 and −262 bp relative to the translation start site. The functionality of this potential binding site was verified by EMSA using as the probe a double-stranded oligonucleotide that contained the PPRE identified in the cav-1 promoter. Indeed, this probe originated a pattern of binding upon incubation with THP-1 nuclear extracts. Moreover, the observed binding was increased when THP-1 macrophages were treated with the PPARγ agonist rosiglitazone. This suggests that the effect of rosiglitazone on cav-1 expression may be mediated by transcription activation upon binding of PPARγ/RXR heterodimers to the newly identified PPRE in the promoter of the cav-1 gene.

Our results do not rule out the possibility that other mechanisms may also participate in cav-1 induction by rosiglitazone. For example, PPARs can also regulate gene transcription in a DNA binding-independent manner by interfering with the activities of several transcription factors, such as NF-κB, AP-1, or STAT-1 (10). Obviously, more studies are needed to fully elucidate the mechanism or mechanisms that mediate the inductive effect of rosiglitazone on cav-1 expression.

In conclusion, in this study we demonstrate that PPAR activators, such as the PPARγ agonist rosiglitazone, increase the expression of cav-1 in macrophages. This effect seems to be mediated by PPAR activation, possibly by the binding of activated PPAR to a new PPRE identified in the cav-1 promoter. However, other mechanisms may also participate in the increase in cav-1 expression after rosiglitazone treatment.

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