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Effects of rosiglitazone and atorvastatin on the expression of genes that control cholesterol homeostasis in differentiating monocytes

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Abbreviations:
ABC, ATP-binding cassette transporter
apo E, apolipoprotein E
ATV, atorvastatin
LXR, liver X receptor
oxLDL, oxidized LDL
PPAR, peroxisome proliferator-activated receptor
ROSI, rosiglitazone
SR-A, scavenger receptor class A
SR-BI, scavenger receptor class B type I
TC, total cholesterol

ABSTRACT

We studied the effects of 5 μM atorvastatin, 2 μM rosiglitazone and their combination on intracellular cholesterol levels and on the expression of genes controlling cholesterol trafficking in human monocytes during their differentiation into macrophages. Our results show that treatment with rosiglitazone caused an increase in CD36 mRNA and protein levels (2.7- and 2.9-fold, $P < 0.001$), but significantly induced the expression of most genes related to cholesterol efflux: ABCA1 mRNA (23%, $P < 0.05$) and protein (2.4-fold, $P < 0.05$), apo E protein (2.4-fold, $P < 0.05$), caveolin-1 mRNA (2.6-fold, $P < 0.001$) and SR-BI mRNA (1.9-fold, $P < 0.001$) and protein (3-fold, $P < 0.01$). As a consequence, rosiglitazone treatment reduced intracellular free cholesterol levels by 22% ($P < 0.01$). Treatment with 5 μM atorvastatin caused the opposite effect on the expression of cholesterol efflux-related genes, which was generally reduced: ABCA1 mRNA (71%, $P < 0.05$), apo E mRNA (46%, $P < 0.001$) and protein (5.6-fold, $P < 0.001$), and CYP27 mRNA (15%, $P < 0.05$). Despite these reductions, intracellular total and free cholesterol levels were also reduced by 30% ($P < 0.01$), an effect that can be attributed to the inhibition of de novo cholesterol synthesis by the statins. The combination of rosiglitazone with atorvastatin attenuated CD36 induction, and caused reductions similar to those caused by the statin alone on the expression of genes involved in cholesterol efflux and on intracellular cholesterol levels.

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1. Introduction

One of the earliest events in the pathogenesis of atherosclerosis is the adherence of monocytes to the endothelium, followed by their migration into the vessel wall and their differentiation into macrophages [1,2]. Intracellular lipid accumulation leading to foam cell formation is mediated by a group of scavenger receptors, mainly scavenger receptor A (SR-A) and CD36 [3]. The unregulated cholesterol accumulation in monocyte/macrophages due to their scavenger receptors is balanced by the expression of proteins that function in cholesterol efflux, such as the ATP-binding cassette transporter ABCA1 [4]. Furthermore, the conversion of cholesterol into more polar metabolites by the sterol 27-hydroxylase (CYP27) pathway also contributes to the elimination of cholesterol from these cells [5], and apolipoprotein E (apo E) facilitates reverse cholesterol transport by acting as an acceptor of free cholesterol effluxed from peripheral cells [6]. Finally, the scavenger receptor class B type I (SR-BI) interacts with both LDL and HDL to promote the bidirectional exchange of free or unesterified cholesterol (FC) between these lipoproteins and SR-BI-expressing cells [7].

Statins are inhibitors of hydroxymethyl-glutaryl-CoA reductase, the committed step in cholesterol biosynthesis, widely used in the treatment of hypercholesterolemia. Several large-scale intervention trials demonstrated that statins reduce the risk of fatal or nonfatal coronary events, cardiovascular mortality and all-cause mortality [8–11]. These clinical benefits may be attributed to the cholesterol-lowering effect of the statins; however, evidence suggests that pleiotropic lipid-independent mechanisms may also contribute to their antiatherosclerotic effect [12]. Despite these beneficial effects, there is still a need for more effective strategies, such as combination of statins with agonists of peroxisome proliferator-activated receptors (PPAR), to improve the results of the current therapies.

PPARs act as ligand-activated transcription factors that, after forming heterodimers with retinoid X receptors (RXRs), bind to specific response elements located in the promoter region of several regulated genes. The subfamily member PPAR-γ is primarily found in adipose tissue and in arterial wall cells such as endothelial cells, vascular smooth muscle cells and monocytes/macrophages, where it modulates lipid metabolism [13]. There is increasing clinical evidence that PPAR-γ agonists exert direct antiatherosclerotic effects [14]. For example, the thiazolidinedione rosiglitazone has been shown to reduce carotid intima-media thickness, a marker for coronary artery disease, in non-diabetic patients [15].

Given the beneficial effects of both statins and PPAR-γ agonists at multiple stages in the atherosclerotic process, their combination may be a promising approach. In fact, the administration of rosiglitazone in combination with atorvastatin has proven safe and effective in controlling glycemia and reducing plasma cholesterol in patients with type 2 diabetes [16]. More recently, Corti et al. [17] demonstrated that a selective PPAR-γ agonist has additional effects on plaque regression in combination with simvastatin in a rabbit model of atherosclerosis. Our previous results also show that the combination of rosiglitazone and atorvastatin has beneficial effects in preventing cholesteryl ester accumulation in differentiated human macrophages exposed to modified LDL [18].

Interestingly, most of the genes that modulate intracellular cholesterol content, such as CD36, SR-A, ABCA1, CYP27 and apo E, are regulated during the differentiation of monocytes into macrophages [19–24]. Moreover, the pharmacological effects of PPAR agonists and statins on these or other related genes in macrophages may depend on the status of differentiation of the cell [25,26]. In addition, during the atherogenesis process, continuous infiltration and differentiation of monocytes occur at the sites of lesion development. Therefore, the objective of the present study was to evaluate the effects of atorvastatin and rosiglitazone, as well as their combination, on the expression of genes controlling cholesterol homeostasis in cultured monocytes during the early stages of their maturation into macrophages. These cells resemble the monocytes that migrate through the subendothelial matrix and begin to differentiate into macrophages during the initial phases of atherogenesis [26].

2. Materials and methods

Pure atorvastatin (calcium salt) was provided by Parke-Davis, Inc., Ann Arbor, USA (now Pfizer), and rosiglitazone by GlaxoSmithKline (Worthing, UK). Cell culture reagents were from GIBCO, Invitrogen Corporation (Paisley, UK), with the exception of fetal bovine serum, which was purchased from Sigma–Aldrich (St. Louis, USA). Ultraspect was obtained from Biotecx (Houston, USA). RT-PCR reagents were from Invitrogen (Gaithersburg, USA), except for the random hexamers and specific primers, obtained from Roche Diagnostics (Indianapolis, USA), and α-[^{32}P]dATP, from Amersham Biosciences (Barcelona, Spain). Antibodies against SR-BI and ABCA1 were from Novus Biologicals (Littleton, USA), against apo E from Biogenesis (Poole, UK), against caveolin-1 from BD Transduction Laboratories (Oxford, UK) and anti-β-tubulin antibody was from Sigma–Aldrich. Other general chemicals were obtained from commercial sources and were of analytical grade.

2.1. Cell culture

THP-1 monocytoid cells were obtained from European Collection of Cell Cultures (ECACC), and maintained in RPMI 1640 medium with 25 mM Hepes Buffer, 10% fetal bovine serum, 1% L-glutamine 200 mM, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2. Monocytes were exposed to complete medium supplemented with 50 ng/ml PMA in the absence or in the presence of 5 μM atorvastatin, 2 μM rosiglitazone or both, for 24 or 48 h. Drug solutions were prepared in DMSO, and the final DMSO concentration was 0.1%. Cell viability, determined by measuring the ability of THP-1 cells to reduce MTT [27], was not reduced, and cell morphology was not altered in the presence of the drugs in any of the conditions tested.

2.2. RNA preparation and analysis

Levels of specific mRNAs were assessed by the reverse transcription-polymerase chain reaction (RT-PCR) under

quantitative conditions. Complementary DNA was synthesized, in a total volume of 20 μl, from RNA samples by mixing 0.5 μg of total RNA, 125 ng of random hexamers in the presence of 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 200 U Moloney murine leukemia virus reverse transcriptase, 20 U RNAsin, 0.5 mM of each dNTP (Sigma), and 50 mM Tris–HCl buffer (pH 8.3). 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 reaction contained 5 μl of the RT reaction, 1.2 mM $MgCl_2$, 200 μM dNTPs, 0.25 μCi [^{32}P]dATP (3000 Ci/mmol), 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 DNA polymerase were separated from primers and dNTPs by using a layer of paraffin (reaction components contact only when paraffin fuses, at 60 °C). The sequences of the forward and reverse primers used for PCR amplification, the length of the PCR product, the number of cycles used in the PCR, and the linear range for the amplification is given in Table 1. PCR was performed in an MJ Research Thermocycler equipped with a peltier system and temperature probe. Preliminary experiments were carried out using different numbers of cycles to determine the linear conditions of PCR amplification for all the genes studied, as shown in Table 1.

[^{32}P]dATP was used in the PCR to produce a radioactive product that could be detected with great sensitivity during the exponential phase of the reaction. After an initial denaturation for 1 min at 94 °C, PCR was performed for the indicated number of 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 were electrophoresed on a 1-mm-thick 5% polyacrylamide gel. Radioactive bands were quantified by video-densitometric scanning (Vilbert Lourmat Imaging). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*gadph*).

2.3. Western blot analysis

Protein extracts (50 μg) from control and treated cells were subjected to 7–12% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to Immobilon polyvinylidene diflouride transfer membranes (Millipore Corp., Bedford, USA). The membranes were blocked 1 h at room temperature in phosphate saline buffer containing 0.1% Tween 20 (PBST) in the presence of 5% nonfat dry milk, and immunological detection was performed using a mouse monoclonal antibody against apo E (dilution 1:500, overnight at 4 °C), a rabbit polyclonal antibody raised against SR-BI (dilution 1:675, 1 h at room temperature), ABCA1 (dilution 1:500, overnight at 4 °C) or caveolin-1 (dilution 1:5000, 1 h at room temperature) [28]. Secondary antibodies were added for 1 h at room temperature, and detection was achieved using the enhanced chemiluminiscence (ECL) detection system (Biological Industries, Israel). Blots were also incubated with a monoclonal antibody raised against β-tubulin (dilution 1:2000), used as a control of equal abundance of protein in the samples. Size of detected proteins was estimated using protein molecular-mass standards (BioRad, Hercules, USA).

Table 1 – Primers used for the polymerase chain reaction (PCR)

Gene sequence of primers (5′–3′)	Product length (bp)	Number of cycles	Linear cycle range
CD36 Forward: CTGTGACCGGAACTGTGGGCT Reverse: GAAGATGGCACCATTGGGCTG	361	18	18–23
SR-A Forward: TCCTCAGTCCAGGGACATGGG Reverse: CGATCTTTTTCACCCGGG	362	18	18–23
ABCA1 Forward: GGAGGCAATGGCACTGAGGAA Reverse: CCTGCCTTGTGGCTGGAGTGT	181	18	18–23
Apo E Forward: TGCTGGTCACATTCCTGGCAG Reverse: GTGACCTGGGAGCTGAGCAGC	202	18	18–23
Caveolin-1 Forward: ACAAGCCCAACAACAAGGCCA Reverse: GAGGGCAGACAGCAAGCGGTA	245	22	20–25
CYP27 Forward: GCCATGGGCAGCCTGCCTGA Reverse: CTTGCGAGGAGTAGCTGCATC	502	23	20–25
SR-BI Forward: ACGACACCGTGTCCTTCCTCG Reverse: CGGGCTGTAGAACTCCAGCGA	509	19	18–23

Primers' sequences, length, number of cycles and linear range for each of the studied genes.

2.4. Flow cytometric analysis for surface expression of CD36

Immunofluorescent flow cytometric analysis was carried out by using a phycoerythryn (PE)-conjugated mouse monoclonal antibody against CD36 (PharMingen, BD Biosciences), as previously described [18]. Briefly, the cells were washed and removed from the dish by incubation with PBS containing 0.5% BSA and 2 mM EDTA. The cell suspension was centrifuged (10 min, at 1000 × *g*, 4 °C), the pellet was re-suspended and incubated with PE-conjugated antibody against CD36 for 30 min at 4 °C in the dark. Then they were washed with ice-cold PBS and analysed with a flow cytometer (Epics XL) equipped with an air-cooled argon-ion laser that excited the fluorescence probe at 488 nm.

2.5. Cellular cholesterol contents

After treatments, cells were scraped in PBS and sonicated with a Branson sonifier 450, set at 25 W, three times during 10 s. An aliquot of the lysate was used to determine cell protein by the method of Bradford [29]. An internal cholestanol standard was added to the rest of the cell lysate, lipids were extracted with chloroform/methanol (2:1, v/v) and total cholesterol was determined by gas–liquid chromatography, as previously described [30].

2.6. Statistical analysis

Data are presented as mean ± standard deviation. An analysis of variance (ANOVA), combined with the Student–Newman–Keuls test, was used to evaluate the statistical significance of the differences. The computer programme GraphPad Instat was used for the calculations.

3. Results

3.1. Effect of drug treatment on the expression of genes involved in the uptake of modified LDL

Human THP-1 monocytes were treated with 5 μM atorvastatin, 2 μM rosiglitazone or a combination of both agents in the presence of the differentiating agent PMA for 24 h. As shown in Fig. 1, the expression of the two main scavenger receptors, SR-A and CD36, was not affected by atorvastatin exposure, compared with cells treated with PMA alone. On the other hand, rosiglitazone significantly induced CD36 both at the mRNA and protein level (2.7- and 2.9-fold increase, $P < 0.001$), without affecting SR-A. When atorvastatin was combined with rosiglitazone, the inductive response on CD36 cell surface expression was reduced by 22% ($P < 0.01$ versus rosiglitazone alone, Fig. 1B).

3.2. Effect of treatment on the expression of genes involved in cholesterol efflux

Fig. 2 shows the effects of the drugs on the expression of genes related to the elimination of cellular cholesterol. Treatment with atorvastatin significantly reduced the expression of ABCA1 (71%, $P < 0.001$), apo E (46%, $P < 0.001$) and CYP27 (15%, $P < 0.05$), while rosiglitazone caused an increase in the mRNA levels of these genes (ABCA1, 23% $P < 0.05$; apo E, 17%, ns; caveolin-1, 2.6-fold increase, $P < 0.01$). On the other hand, treatment with rosiglitazone caused a marked reduction in CYP27 mRNA levels (68% decrease, $P < 0.001$).

The combination of atorvastatin and rosiglitazone caused a decrease in ABCA1 and apo E mRNA of a magnitude similar to that caused by atorvastatin (62 and 33%, respectively). However, in the case of CYP27 mRNA levels, the effect of the combination was greater than that of each agent alone. Thus, in the presence of atorvastatin and rosiglitazone, the mRNA levels of CYP27 were reduced by 80%, which represents a decrease of 38% ($P < 0.05$) on the effect caused by rosiglitazone alone.

Changes in mRNA expression were also observed at the protein level (Fig. 2B, D and F). Atorvastatin, alone or combined with rosiglitazone, markedly reduced ABCA1 and apo E protein levels, while rosiglitazone increased those of ABCA1 (2.4-fold, $P < 0.05$), apo E (2.4-fold, $P < 0.01$) and caveolin-1 (3.8-fold).

3.3. Effect of treatment on the expression of SR-BI

Fig. 3 shows the mRNA and protein levels of SR-BI, a receptor which promotes the bidirectional flux of cholesterol between cells and lipoproteins [7]. Treatment with atorvastatin caused a moderate increase in SR-BI mRNA levels (36%, $P < 0.05$), while rosiglitazone and the combination increased them to a much greater extent (around two-fold, $P < 0.001$). Similarly, rosiglitazone and the combination of rosiglitazone plus atorvastatin greatly increased SR-BI protein levels (3- and 3.5-fold, respectively, $P < 0.01$).

3.4. Effect of treatments on intracellular cholesterol contents

THP-1 cells were treated with PMA in the presence or in the absence of the drugs under study for 24 h (Fig. 4A and C) and with the drugs alone for an additional 24 h period (Fig. 4B and D). The 24 h-treatments did not cause significant effects on intracellular cholesterol accumulation. When macrophages were treated for 48 h, there was a decrease in total cholesterol levels that could be mainly attributed to differences in free cholesterol levels. As shown in Fig. 4D, atorvastatin, rosiglitazone and the combination of both agents reduced intracellular free cholesterol levels by 29, 22 and 32%, respectively.

4. Discussion

In the earliest stages of atherogenesis, monocytes are recruited to the vascular wall, where they differentiate to macrophages and lipid-laden foam cells. Throughout atherosclerosis development, inflammatory cytokines and oxidized LDL in atheroma trigger chemotaxis, so that there is a continuous process of monocyte migration and differentiation into the inflammatory lesions. However, monocytes should not be regarded as mere precursors of macrophages, as their infiltration and accumulation correlate with smooth muscle

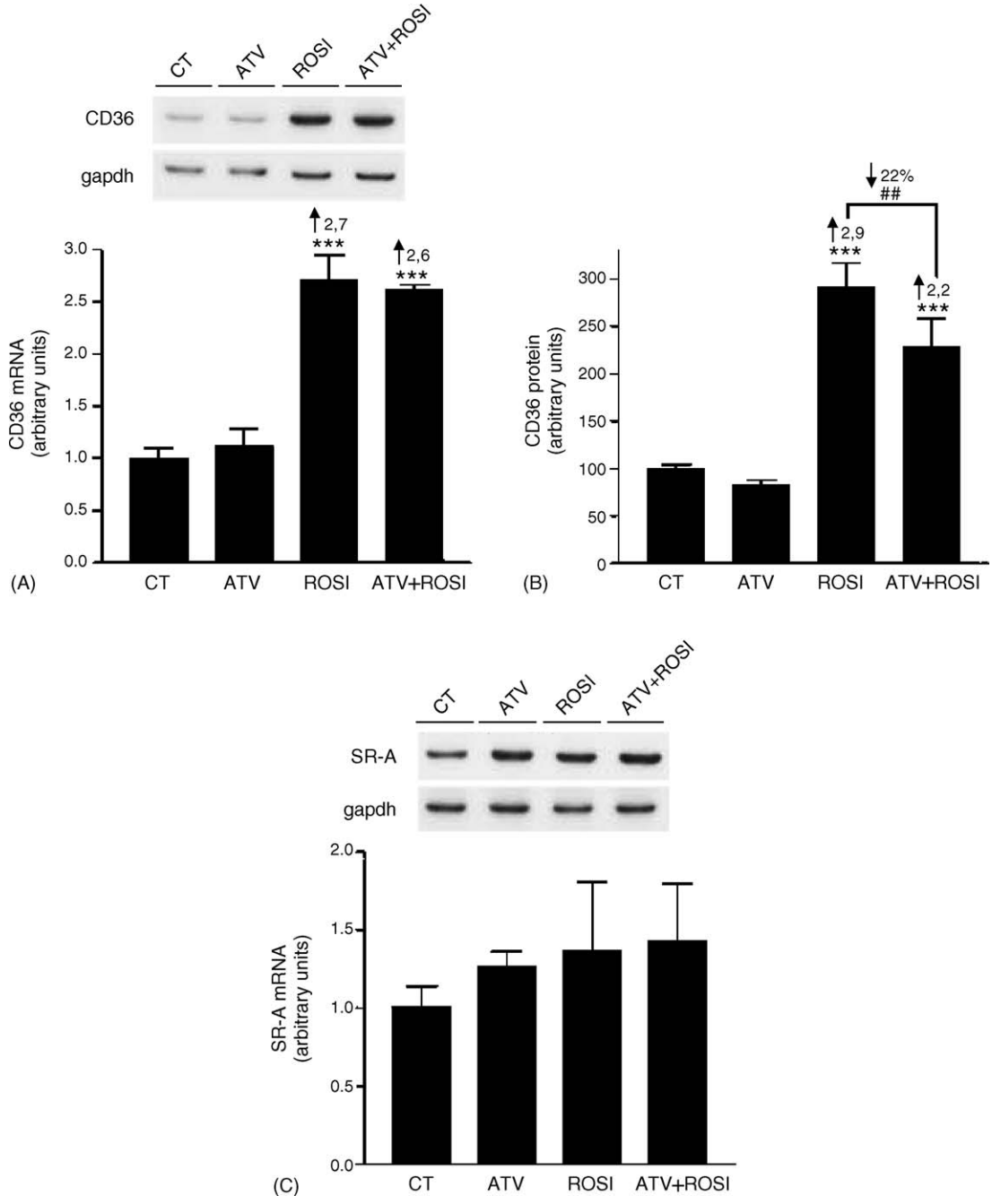

Fig. 1 – Effect of drug treatments on the expression of genes and proteins involved in cholesterol uptake. THP-1 monocytes were treated with PMA in the absence (CT) or in the presence of 5 μM of atorvastatin (ATV), 2 μM rosiglitazone (ROSI) or both (ATV + ROSI) during 24 h. Analysis of CD36 (A) and SR-A (C) mRNA levels. 0,5 μg of total RNA were analyzed by RT-PCR. Representative autoradiograms and quantifications of the gapdh-normalized mRNA levels are shown. Data are expressed as mean ± standard deviation (S.D.) of three independent experiments run in duplicate. Analysis of CD36 (B) surface protein expression by flow cytometry, using a phycoerithrin (PE)-conjugated antibody against CD36. Data are expressed as the percentage of mean fluorescence intensity ± S.D. of three independent experiments run in duplicate. $^{***}P < 0.01$ compared with corresponding control cells. $\#\#P < 0.01$ compared with rosiglitazone-treated cells.

cell proliferation and play an important role in neointimal hyperplasia [31]. Therefore, we were interested in investigating the effects of atorvastatin and rosiglitazone (alone or combined) on the expression of genes related to cholesterol trafficking and on cholesterol contents in monocytes during their early stages of differentiation to macrophages.

Rosiglitazone treatment caused a general increase in the expression of cholesterol efflux-related genes, while causing a marked increase in CD36 mRNA and protein levels. This effect has been already described, as it is well known that CD36 expression is regulated by PPAR-γ activation [18,32]. According to our results obtained in mature macrophages [18], the

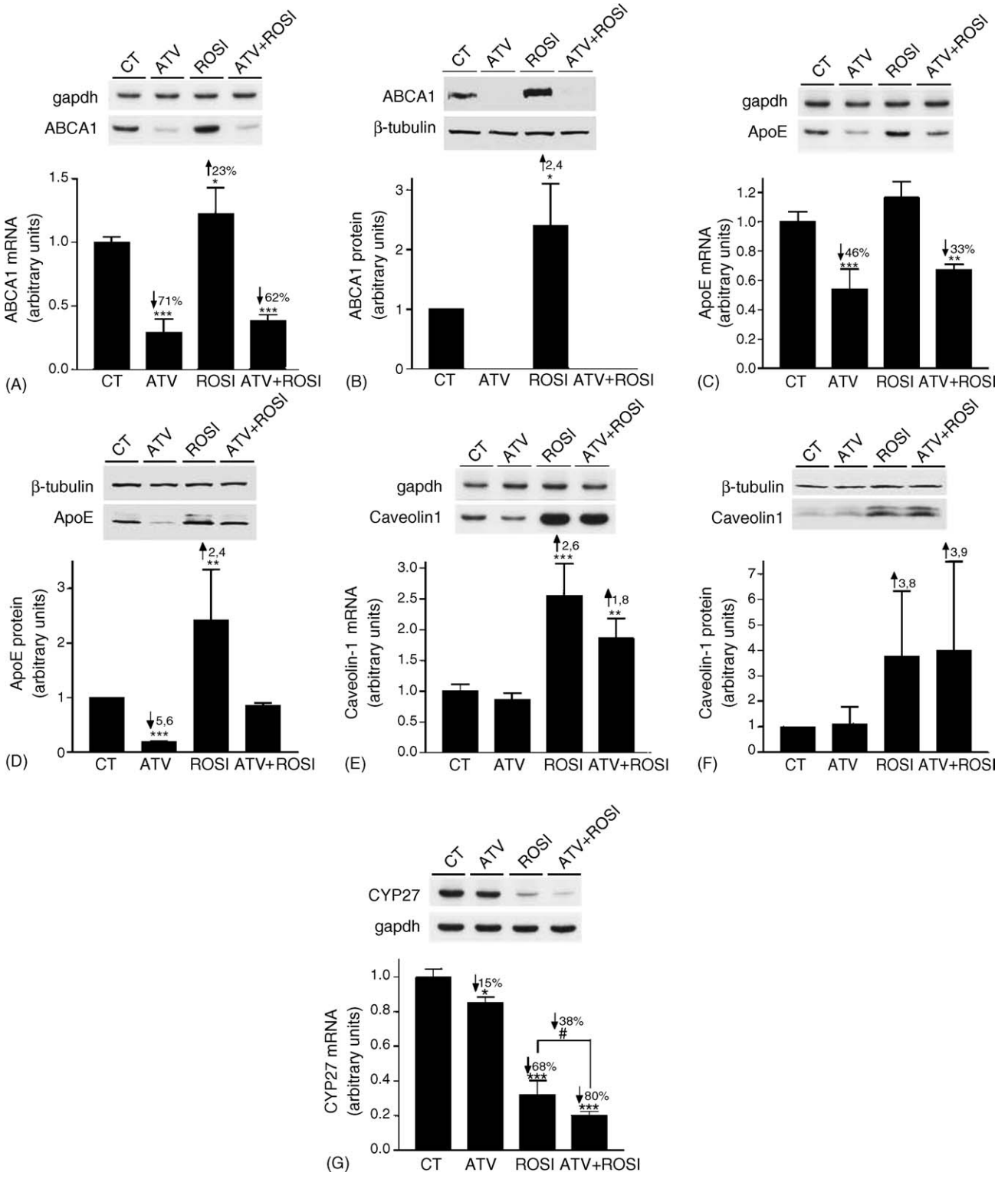

Fig. 2 – Effect of treatments on the expression of genes and proteins involved in cholesterol efflux. THP-1 monocytes were treated with PMA in the absence (CT) or in the presence of 5 μM of atorvastatin (ATV), 2 μM rosiglitazone (ROSI) or both (ATV + ROSI) during 24 h. Analysis of ABCA1 (A), apo E (C), caveolin-1 (E) and CYP27 (G) mRNA levels. A 0.5 μg of total RNA were analyzed by RT-PCR. Representative autoradiograms and quantifications of the gapdh-normalized mRNA levels are shown. Data are expressed as mean ± standard deviation (S.D.) of three independent experiments run in duplicate. Analysis of ABCA1 (B), apo E (D) and caveolin-1 (F) protein expression by Western Blot. Whole protein extracts (30–50 μg) were resolved in 7 or 12% SDS-polyacrilamide gel. To show equal loading of protein, β-tubulin signal from the same blots are included. A representative autoradiograms and the quantifications are shown. Data are expressed as mean ± S.D. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.01$ compared with corresponding control cells. $^{\#}P < 0.05$ compared with rosiglitazone-treated cells.

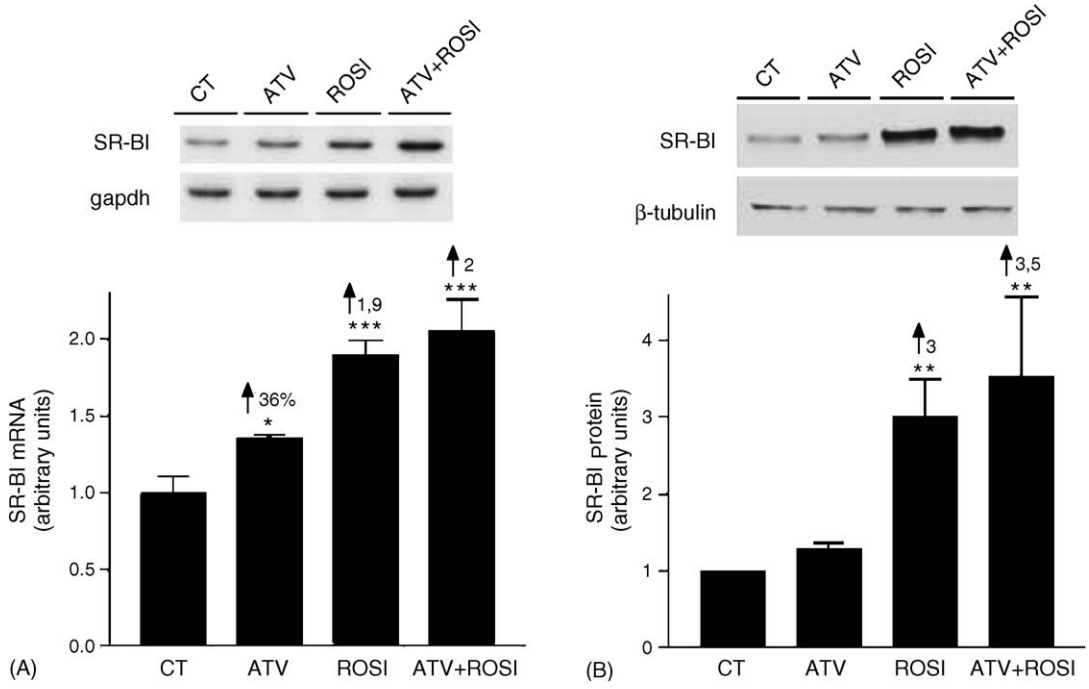

Fig. 3 – Effect of treatments on SR-BI gene and protein expression. THP-1 monocytes were treated with PMA in the absence (CT) or in the presence of 5 μM of atorvastatin (ATV), 2 μM rosiglitazone (ROSI) or both (ATV + ROSI) during 24 h. Analysis of SR-BI (A) mRNA levels. 0.5 μg of total RNA were analyzed by RT-PCR. Representative autoradiogram and quantification of the gapdh-normalized mRNA levels are shown. Data are expressed as mean ± standard deviation (S.D.) of three independent experiments run in duplicate. Analysis of SR-BI (B) protein expression by Western Blot. Whole protein extracts (30 μg) were resolved in 7% SDS-polyacrilamide gel. To show equal loading of protein, β-tubulin signal from the same blots are included. A representative autoradiogram and the quantification are shown. Data are expressed as mean ± S.D. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.01$ compared with corresponding control cells.

increase in CD36 was attenuated when the drug was combined with atorvastatin (Fig. 1B). Divergent results have been obtained regarding CD36 expression in monocytes/macrophages after a combined treatment with a statin and a PPAR-γ agonist [33,34]. Though we have not found a significant decrease in CD36 expression after treatment with atorvastatin alone, other authors have described that statins down-regulate CD36 expression in monocytes and macrophages [26,35]. It is possible that in our model, and at the concentration of atorvastatin assayed, the inhibitory effect is only evidenced when basal CD36 expression is increased by the addition of a PPAR-γ activator.

Regarding the genes involved in cholesterol efflux, rosiglitazone increased ABCA1 and apo E expression, effects that involve liver receptor X (LXR) activation [36,37]. Treatment with rosiglitazone, alone or combined with atorvastatin, also increased SR-BI mRNA and protein levels. An increase in SR-BI expression by PPAR-γ has also been reported in differentiated primary human macrophages [38] and in macrophage-like Kupffer cells [39]. However, in human non-differentiated monocytes treatment with rosiglitazone 20 nM did not affect SR-BI expression, while treatment with PPAR-α agonists caused an increase [38]. These divergent results could be explained by the fact that at the concentration of rosiglitazone used in our study a certain degree of PPAR-α activation could occur [40] that may be responsible of the observed SR-BI increase. The mRNA and protein levels of caveolin-1 were also increased after treatment with rosiglitazone, in keeping with our previous report suggesting the existence of a PPRE in the caveolin-1 promoter [28]. In contrast, the mRNA levels of CYP27 were reduced by rosiglitazone treatment. Quinn et al reported that PPAR-γ agonists increase CYP27 mRNA expression in fully differentiated macrophages, and that THP-1 differentiation (either by PMA or by an alternative method) repress CYP27 expression [41]. Therefore, it is conceivable that the decrease in CYP27 expression found in our study after rosiglitazone exposure may be related to the differentiating effect of PPAR-γ agonists on monocytic cells [42].

On the other hand, atorvastatin (alone or combined with rosiglitazone) reduced the expression of most cholesterol-efflux-related genes. Thus, the mRNA and protein levels of ABCA1 and apo E were significantly reduced upon incubation with atorvastatin (Fig. 2A–D). A reduction of ABCA1 mRNA expression by different statins in RAW246.7 and mouse peritoneal macrophages has been recently reported [43,44]. A mechanism that may explain this reduction is related to the inhibitory effect of the statins on the synthesis of oxysterols, which are LXR ligands. This would lead to a decrease in the expression of LXR target genes, such as ABCA1 or apo E [37,45]. A second mechanism which might serve to explain the inhibitory effect of atorvastatin on ABCA1 would be related to the activation of SREBP2 due to the reduction of the de novo cholesterol synthesis just as in vascular endothelial cells, where SREBP2 activation down-regulates ABCA1 [46]. The

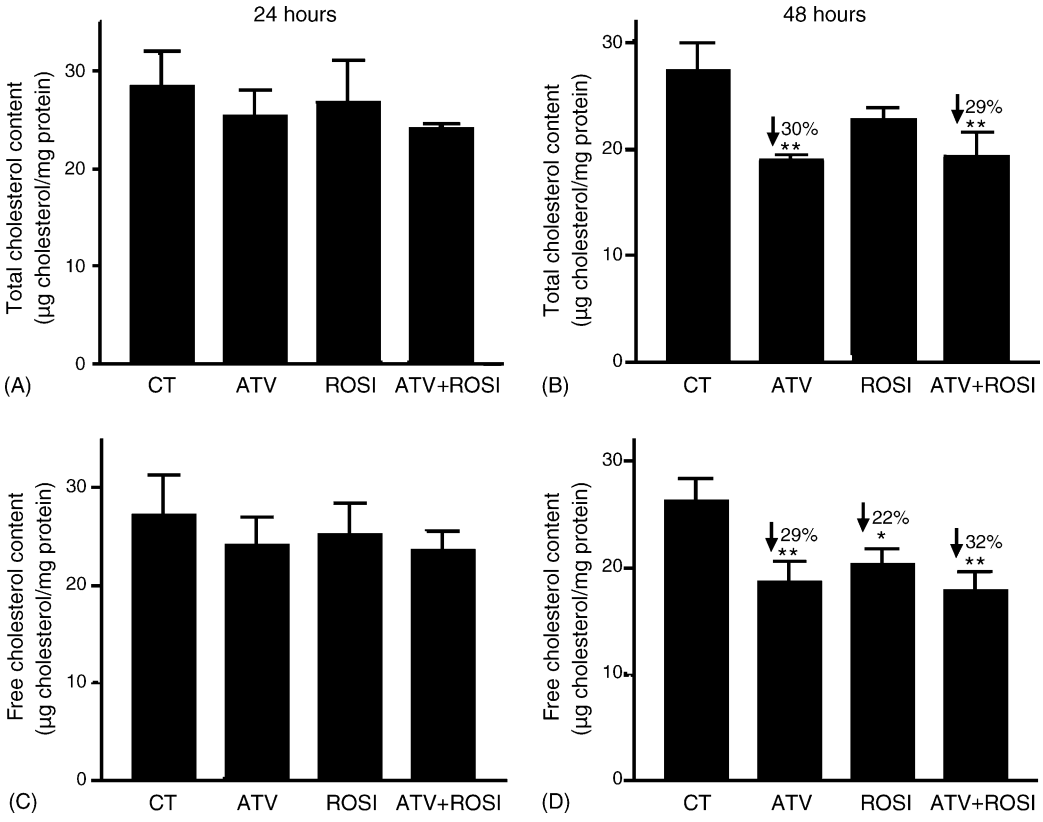

Fig. 4 – Effect of treatments on intracellular cholesterol contents. THP-1 monocytes were treated with PMA in the absence (CT) or in the presence of 5 μM of atorvastatin (ATV), 2 μM rosiglitazone (ROSI) or both (ATV + ROSI) during 24 h (A and C) or 48h (B and D). After treatments, cells were washed, cellular lipid was extracted and total cholesterol (TC) (A and B), and free cholesterol (FC) (C and D) mass was determined by gas–liquid chromatography. Results are expressed as μg sterol/mg cellular protein, and are the mean ± S.D. of three independent experiments performed in duplicate wells. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with corresponding control cells.

increase in SR-BI mRNA caused by atorvastatin (Fig. 3A) may also be a consequence of SREBP2 activation, as the promoter region of the SR-BI gene is transactivated by SREBP-2 [47].

Regarding intracellular cholesterol, rosiglitazone treatment reduced total and free cholesterol levels despite the observed increase in CD36 expression. As described by other authors [13], the increase in CD36 caused by PPAR-γ agonists may be counteracted by the induction of the expression of genes that drive cholesterol efflux (ABCA1, apo E, caveolin-1 and SR-BI), resulting in a reduction of intracellular cholesterol. Atorvastatin treatment also caused a reduction in total cholesterol levels, mainly due to a significant drop in intracellular free cholesterol. These effects may not be explained by the increase in the expression of genes involved in cholesterol efflux pathways, which would lead to an accumulation of intracellular sterols. Rather, the effect may be related to inhibition of de novo cholesterol synthesis by the statin. This behaviour is the opposite of that observed in our previous study [18], where extensive lipid loading probably prevented the inhibition of HMG-CoA reductase activity by atorvastatin.

Finally, the combination of atorvastatin and rosiglitazone caused a decrease in intracellular total and free cholesterol of a magnitude similar to that obtained for each agent alone. Combination therapy, which is already used in the treatment of other complex pathologies such as hypertension, is a promising future approach for the treatment and prevention of atherosclerosis. In fact, though statin monotherapy reduces cardiovascular risk by approximately 30%, there is still a need for more effective therapies that may improve the current standards. In this setting, the combination of a statin and PPAR-gamma agonist is an interesting approach, as both agents exert pleiotropic effects that may influence plaque development [17]. Specifically, in patients with type 2 diabetes, where insulin resistance and dyslipidemia coexist, rosiglitazone in combination with atorvastatin caused beneficial effects on glycemic control and plasma lipid parameters [16]. Our results obtained in differentiated macrophages [18] and in monocytes during their differentiation to macrophages show that, in addition to these effects, the combination of rosiglitazone and atorvastatin may directly influence foam cell formation. These experimental findings, if confirmed in humans, may contribute to show the antiatherogenic potential of this combination.

In summary, the results of the present study suggest that statins and PPAR-γ ligands regulate the expression of genes related to cholesterol import/export in the earliest steps of macrophage differentiation, and reduce their cholesterol content even in the absence of lipid loading. Both the differentiation status of the cell and exogenous lipid exposure

may account for the different behaviour of these cells in comparison with mature lipid-loaded macrophages. However, in both cases treatment with atorvastatin, rosiglitazone or the combination of both agents reduce intracellular cholesterol accumulation.

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