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Reduction of intracellular cholesterol accumulation in THP-1 macrophages by a combination of rosiglitazone and atorvastatin

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Abstract

Rosiglitazone and atorvastatin combination therapy has beneficial effects on both glycemic control and plasma lipid levels in type 2 diabetic patients. In the present study, we sought to determine whether this combination can also exert direct antiatherosclerotic effects in macrophages. Our results show that 2 μM rosiglitazone, alone or combined with 5 μM atorvastatin, significantly upregulated the expression of the ATP-binding cassette transporter ABCA1 and of the class B scavenger receptor CLA-1 (CD36 and LIMPII analog), both involved in cholesterol efflux from macrophages. On the other hand, the combination with atorvastatin attenuated the inductive response elicited by rosiglitazone alone on CD36 mRNA (34%, $P < 0.05$) and protein (16%, $P < 0.05$), while the uptake of oxidized low density lipoprotein (LDL) remained unaffected. When we examined the effects of the drugs on acetyl-LDL-induced cholesterol accumulation, we found that only the combination of atorvastatin with rosiglitazone caused a net depletion in the cholesteryl ester content of macrophages (35%, $P < 0.05$). Our data suggest that this reduction was not mediated by effects on proteins that regulate cholesterol flux, but it may be related to the inhibition of cholesteryl ester formation elicited by the statin.

Keywords: Macrophages; Rosiglitazone; Atorvastatin; Cholesterol; CD36; ABCA1; CLA-1

1. Introduction

Atherosclerosis is one of the major vascular complications of diabetes [1], and epidemiological studies have confirmed a strong direct association between cardiovascular diseases and proper glycemic control [2]. Thiazolidinediones, such as rosiglitazone, are increasingly being used in the treatment of type 2 diabetes, due to their insulin-sensitizing effects. The mechanism of action of these compounds involves binding to PPARγ, a member of the nuclear hormone receptor family that heterodimerize with the retinoid X receptor [3]. PPARγ ligands regulate the expression of specific genes in adipocytes, but also in other cell types such as macrophages, where they act as modulators of cell differentiation and lipid metabolism [4,5].

The coexistence of insulin resistance and dyslipidemia in type 2 diabetes, supports the rationale for the use of a combination of insulin sensitizers and lipid lowering drugs. In this regard, a recent clinical study evaluated the efficacy and safety of a combination of rosiglitazone and atorvastatin, an hydroxymethyl-glutaryl-CoA reductase inhibitor or statin, in patients with type 2 diabetes [6]. This study demonstrated the beneficial effects produced by this combination on glycemic control and plasma lipid parameters. However, atherosclerosis is a multifactorial disease of great complexity, and the correction of plasma lipid abnormalities is not the only factor leading to a reduction in atherosclerotic risk. Drugs such as statins, in addition to their hypocholesterolemic properties, can exert a multiplicity of effects on cells of the arterial wall that directly interfere with the atherosclerotic process [7]. Moreover, since both PPARγ ligands [8–11] and statins [12–14]

Abbreviations: ABC, ATP-binding cassette transporter; Ac-LDL, acetylated LDL; apo E, apolipoprotein E; ATV, atorvastatin; CE, cholesteryl ester; CLA-1, CD36 and LIMPII analog; LDL, low density lipoproteins; 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

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regulate cholesterol accumulation in macrophages, their combined administration may also influence foam cell formation.

Therefore, the objective of the present study has been to evaluate whether the combination of rosiglitazone and atorvastatin exerts direct antiatherosclerotic effects on macrophages that can contribute to limit foam cell formation. To this end, we have studied the effect of these drugs, alone or in combination on the expression of an array of genes that control cholesterol balance in macrophages: SR-A and CD36, scavenger receptors involved in the unregulated uptake of modified lipoproteins that leads to the massive accumulation of cholesterol characteristic of foam cells [15], the transporters ABCA1 and ABCG1, which facilitate the efflux of cholesterol and phospholipids to apolipoprotein A-1 [16,17], and CLA-1, the human homologue of SR-BI, which promotes cholesterol efflux to HDL [18]. The expression of apo E, that confers the ability to export cholesterol from macrophages even in the absence of exogenously added acceptors [19], and of sterol 27-hydroxylase (CYP-27), that facilitates the removal of hydrophilic cholesterol metabolites from these cells [20], were also studied. Finally, we have further examined the effects of these drugs on intracellular cholesterol accumulation induced by acetyl-LDL loading.

2. Material and methods

Pure atorvastatin (calcium salt) was provided by Parke-Davis, Inc., Ann Arbor, MI (now Pfizer), and rosiglitazone by GlaxoSmithkline. Cell culture reagents were from Gibco, Invitrogen Corporation, with the exception of fetal bovine serum and 4-beta phorbol 12-beta myristate 13-alpha acetate (PMA), which were purchased from Sigma-Aldrich. Ultraspect was obtained from Biotecx. RT-PCR reagents were from Invitrogen, except for the random hexamers and specific primers, obtained from Roche Diagnostics, and α-[^{32}P]dATP, from Amersham Biosciences. Antibodies against SR-BI and ABCA1 were from Novus Biologicals, against SR-AI from Serotec and anti-β-actin from Sigma-Aldrich. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was from Molecular Probes. Other general chemicals were obtained from commercial sources and were of analytical grade.

2.1. Lipoproteins

LDL (d 1.019–1.063) were prepared from the plasma of healthy donors by sequential ultracentrifugation as previously described [21]. Lipoproteins were dialyzed against phosphate saline buffer (PBS) at 4 °C in the darkness, and their protein concentration was assessed by the method of Bradford [22], using bovine serum albumin as a standard. LDL were acetylated as described by Basu et al. [23]. According to Devaraj et al. [24], LDL were labeled with the fluorescent probe DiI before oxidative modification. DiI-Labeled LDL (DiI-LDL) were prepared as described previously [25]. Briefly, LDL (1 mg/ml) were incubated in the dark at 37 °C for 18 h with 150 μg of DiI in DMSO. DiI-LDL were subsequently reisolated by centrifugation (110,000 × g, 18 h) and dialyzed against PBS, at 4 °C in the dark, for 48 h. LDL and DiI-LDL were oxidized by incubation with 5 μM $CuSO_4$ for 5 h at 37 °C, and dialyzed against PBS (24 h, 4 °C). Lipoproteins were stored at 4 °C protected from light, and used within 15 days.

2.2. Cell culture

THP-1 cells were obtained from European Collection of Cell Cultures (ECACC), and maintained in medium A (RPMI 1640 with 25 mM Hepes Buffer, supplemented with 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. Differentiation of monocytes to macrophages was achieved by exposing the cells to 50 ng/ml PMA during 72 h [26]. Macrophages were washed three times with PBS and incubated in medium A devoid of serum, with 150 μg/ml acetyl-LDL 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.

2.3. RNA preparation and analysis

Total RNA was isolated using the Ultraspec reagent (Biotecx). Relative levels of specific mRNAs were assessed by the reverse transcription-polymerase chain reaction (RT-PCR). Complementary DNA was synthesized from RNA samples by mixing 0.5 μg of total RNA, 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 U Moloney murine leukemia virus reverse transcriptase, 20 U RNAsin and 0.5 mM of each 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 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, Amersham), 1 U 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 sense and antisense primers used for amplification were: *human SR-AI*, 5′-TCCTCAGCTCAG-

GGACATGGG-3′ and 5′-CGATCTCCTTTTTCACCCG-GG-3′, *cd36*, 5′-CTGTGACCGGAACTGTGGGCT-3′ and 5′-GAAGATGGCACCATTGGG CTG-3', *cla-1*, 5'-ACG-ACACCGTGTCCTTCCTCG-3′ and 5′-CGGGCTGTA-GAA CTCCAGCGA-3′, *cyp27*, 5′-GCCATGGGCAGC-CTGCCTGA-3′ and 5′-CTTGCGAGGAGTAGCTGCA-TC-3′, *AbcA1*, 5′-GGAGGCAATGG CACTGAGGAA-3′ and 5′-CCTGCCTTGTGGCTGGAGTGT-3′, *AbcG1*, 5′-CCATGATGGTGTCGGCACATC-3′ and 5′-GCTGGTG-GGCTCATCGAAGAA-3′, *apoE*, 5′-TGCTGGTCACAT-TCCTGGCAG-3′ and 5′-GTGACCTGGGAGCTGAG-CAGC, *gapdh*, 5′-CAGTCCATGCCATCACTGCCA-3′ and 5′-GGTGGAGGAGTGGGTGTCGC-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 21 (*SR-AI* and *cd36*), 23 (*cla-1*), 22 (*cyp-27*), 20 (*AbcA1*), 24 (A*bcG1*) and 18 (*apoE* and *gapdh*) 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 (*SR-AI,* 362 bp, *cd36*, 361 bp, *cla-1*, 509 bp, *cyp-27*, 502, *AbcA1*, 181 bp, *AbcG1* 206 bp, *apoE* 202 bp and *gapdh* 302 bp). Preliminary experiments were carried out with various amounts of cDNA and different number of cycles to determine non-saturating conditions of PCR amplification for all the genes studied. In these conditions, relative quantification of mRNA was assessed by the RT-PCR method [28]. 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 (*gapdh*).

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). Cells were cultured and treated with the drugs as described above. After treatments, 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 resuspended 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 analyzed with a flow cytometer (Epics XL) equipped with an air-cooled argon-ion laser that excited the fluorescence probe at 488 nm.

2.5. Western blot analysis

Protein extracts (50 μg) from control and treated cells were subjected to 7% SDS–polyacrylamide gel electrophoresis. Proteins were then transferred to Immobilon polyvinylidene diflouride transfer membranes (Millipore). 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 rabbit polyclonal antibody raised against SR-BI (dilution 1:675, 1 h at room temperature) or a rabbit polyclonal antibody against ABCA1 (dilution 1:500, overnight at 4 °C). For SR-AI, after blocking with Tween saline buffer (TBS) containing 5% nonfat dry milk, membranes were incubated overnight with a primary antibody (goat polyclonal against human SR-AI, dilution 1:2000) in TBS with 1% nonfat dry milk. Secondary antibodies were added for 1 h at room temperature, and detection was achieved using the enhanced chemiluminiscence (ECL) detection system (Biological Industries). Blots were also incubated with a monoclonal antibody raised against β-actin (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).

2.6. Binding and uptake of DiI-labeled oxLDL

Binding and uptake experiments were performed as described before [29,30] with some modifications. Essentially, THP-1 macrophages were treated for 24 h as described in Section 2.2, and then incubated with DiI-oxLDL (20 μg/ml) for 3 h at 4 °C (binding) or 37 °C (uptake). Cells were then washed with PBS and removed from the plate 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 resuspended in PBS containing 0.5% BSA, and DiI fluorescence was analyzed using an Epics Elite flow cytometer (Coulter Corporation, USA). Excitation of the sample was done using a water-cooled argon-ion laser tuned at 514 nm at 40 mW power. Forward scatter and side scatter from green laser and orange fluorescence (575 nm) were collected. Optical alignment was based on optimized signal from 10 μm fluorescent beads (Flow Check, Coulter Corporation, USA). Specific cellular binding and uptake were calculated by subtracting the respective unspecific values, obtained by coincubation with a 50-fold excess of unlabeled oxLDL.

2.7. 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 [22]. 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 and free cholesterol were determined by gas–liquid chromatography, as previously described [26].

2.8. 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 cholesterol efflux

THP-1 monocytes were fully differentiated into macrophages by PMA exposure during 72 h, and subsequently treated with the drugs under study (5 μM atorvastatin, 2 μM rosiglitazone or a combination of both agents), in the presence of 150 μg/ml acetyl-LDL for 24 h.

None of the drugs under study, neither alone nor combined, caused significant modifications in the mRNA expression of several genes that play a role in the efflux of cholesterol from macrophages: the ABC transporters, ABCA1 and ABCG1, CLA-1, the human homologue of SR-BI, CYP-27 and apo E (Table 1).

Western blot analysis were performed with specific antibodies to CLA-1 and ABCA1 on proteins from control and treated macrophages (Fig. 1). Atorvastatin exposure did not produce significant changes in CLA-1 protein levels, while treatment with rosiglitazone, alone and combined with atorvastatin, upregulated CLA-1 by about 75% ($P < 0.05$). Similarly, ABCA1 was maximally upregulated by rosiglitazone or the combination with the statin (2.2- and 2.4-fold increase, $P < 0.05$).

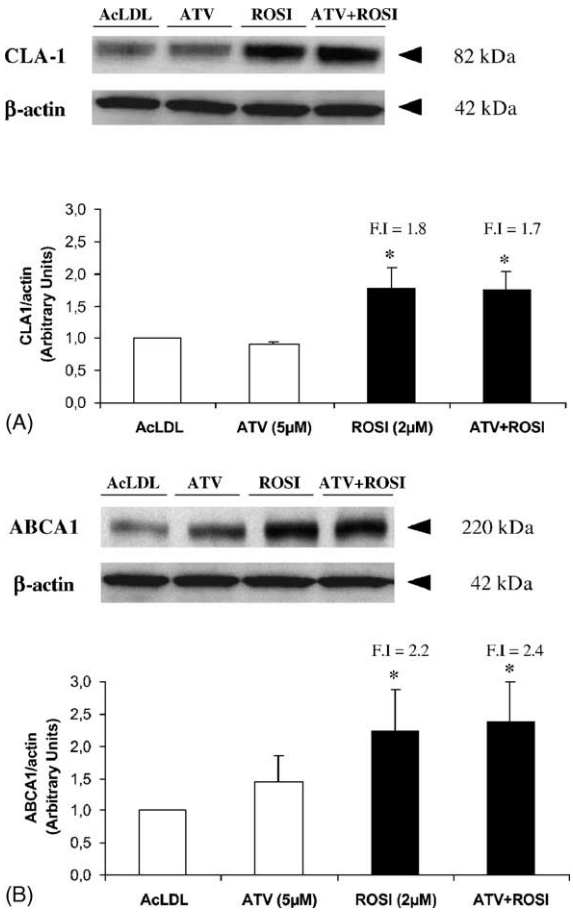

Fig. 1. Effects of 5 μM atorvastatin (ATV), 2 μM rosiglitazone (ROSI) or both on CLA-1 and ABCA1 protein levels in THP-1 macrophages. Cells were simultaneously incubated with acetylated-LDL (150 μg/ml) in the presence or absence of the drugs under study for 24 h and protein extracts were prepared. An equal amount of protein from each sample (50 μg) was resolved by SDS–PAGE and immunoblotted with antibodies against CLA-1, ABCA1 and β-actin. The β-actin immunoblot serves as a control for the amount of protein loaded in each lane. Densitometric analyses from three independent experiments and representative Western blots for each protein are displayed. $^{*}P < 0.05$ compared with cells treated with acetyl-LDL alone.

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

Human SR-AI mRNA expression was not affected by atorvastatin exposure, in comparison with macrophages treated with acetyl-LDL alone; on the other hand, treatment with rosiglitazone or with the combination atorvastatin-rosiglitazone produced modest increases (46 and 39%, respectively, $P < 0.05$) in SR-AI mRNA levels (Fig. 2A). However, when protein levels of this scavenger receptor were examined, we did not found significant modifications in any of the conditions tested (Fig. 2B).

Table 1
Effect of atorvastatin, rosiglitazone and their combination on the mRNA levels of genes involved in cholesterol efflux from macrophages

	ABCA1	ABCG1	CLA-1	Apo E	CYP-27
AcLDL	1.15 ± 0.13	1.08 ± 0.12	0.78 ± 0.26	0.76 ± 0.07	1.95 ± 0.53
ATV	1.05 ± 0.20	1.06 ± 0.16	0.81 ± 0.24	0.75 ± 0.05	2.26 ± 0.51
ROSI	1.34 ± 0.32	1.25 ± 0.23	0.82 ± 0.26	0.74 ± 0.05	1.78 ± 0.66
ATV + ROSI	1.17 ± 0.28	1.08 ± 0.17	0.85 ± 0.28	0.78 ± 0.06	2.19 ± 0.85

Effects of 5 μM atorvastatin (ATV), 2 μM rosiglitazone (ROSI) and the combination of both agents on the mRNA levels of ABCA1, ABCG1, CLA-1, apolipoprotein E (apo E) and sterol 27-hydroxylase (CYP-27) in THP-1 macrophages. Cells were simultaneously incubated with acetylated-LDL (150 μg/ml) in the presence or absence of the drugs under study for 24 h. Total RNA (0.5 μg) was analyzed by RT-PCR, and the quantification of the *gapdh*-normalized mRNA levels are shown. Data are expressed in arbitrary units as mean ± S.D. of three separate experiments performed using different batches of cells and lipoproteins.

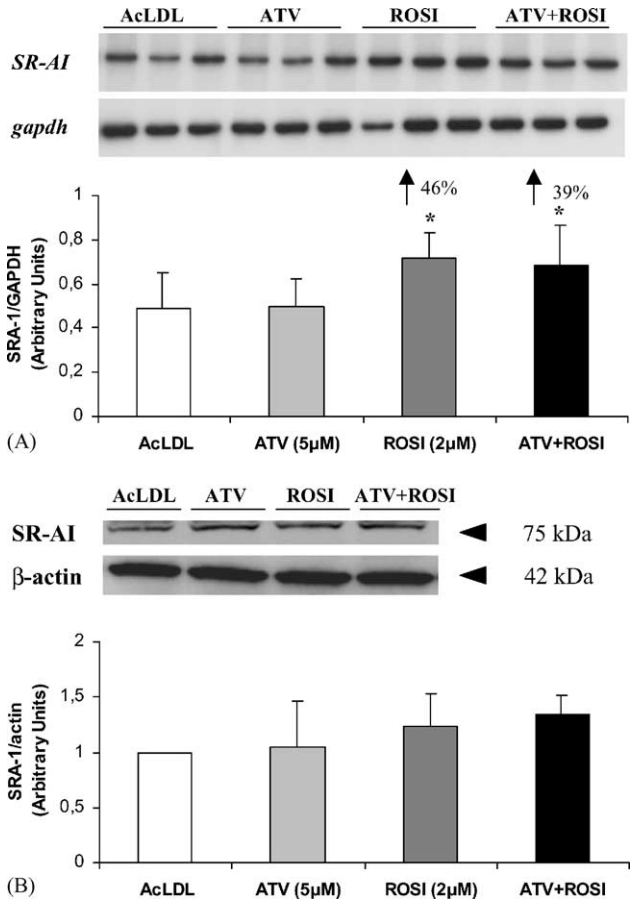

Fig. 2. (A) Effects of 5 μM atorvastatin (ATV), 2 μM rosiglitazone (ROSI) and the combination of both agents on the expression of SR-AI mRNA in THP-1 macrophages. Cells were simultaneously incubated with acetylated-LDL (150 μg/ml) in the presence or absence of the drugs under study for 24 h. Total RNA (0.5 μg) was analyzed by RT-PCR. A representative autoradiogram and the quantification of the gapdh normalized mRNA levels are shown. Data are expressed as mean ± S.D. of three separate experiments performed using different batches of cells and lipoproteins. $^{*}P < 0.05$ compared with cells treated with acetyl-LDL alone. (B) Effects of 5 μM atorvastatin (ATV), 2 μM rosiglitazone (ROSI) and the combination of both agents on SR-AI protein expression in THP-1 macrophages. Cells were simultaneously incubated with acetylated-LDL (150 μg/ml) in the presence or absence of the drugs under study for 24 h and protein extracts were prepared. An equal amount of protein from each sample (50 μg) was resolved by SDS–PAGE and immunoblotted with antibodies against SR-AI and β-actin. The β-actin immunoblot serves as a control for the amount of protein loaded in each lane. Densitometric analyses from three independent experiments and a representative Western blot are displayed.

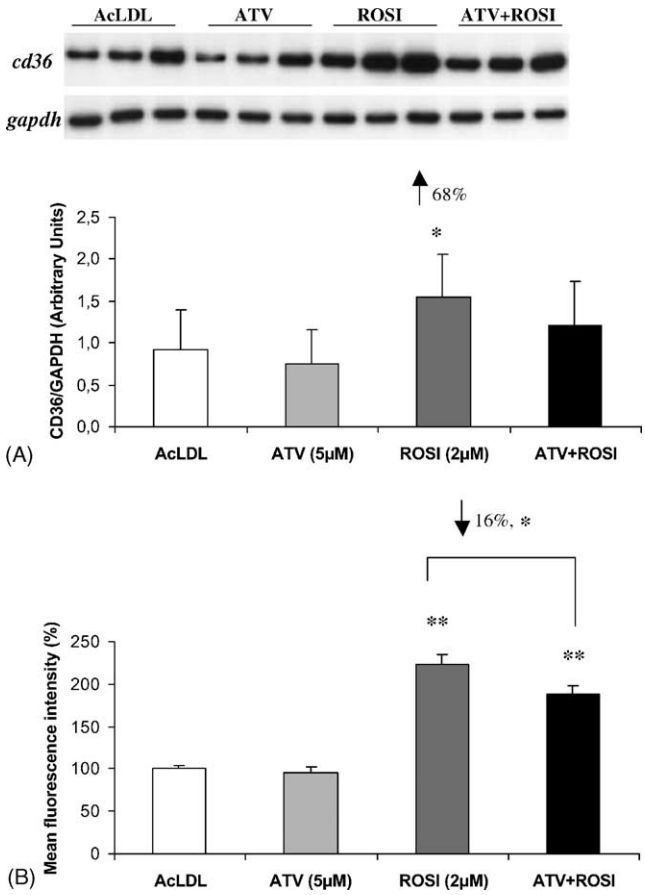

Fig. 3. (A) Effects of 5 μM atorvastatin (ATV), 2 μM rosiglitazone (ROSI) and the combination of both agents on CD36 mRNA levels in THP-1 macrophages. Cells were simultaneously incubated with acetylated-LDL (150 μg/ml) in the presence of vehicle or the drugs under study for 24 h. Total RNA (0.5 μg) was analyzed by RT-PCR. A representative autoradiogram and the quantification of the gapdh normalized mRNA levels are shown. Data are expressed as mean ± S.D. of three separate experiments performed using different batches of cells and lipoproteins. $^{*}P < 0.05$ compared with cells treated with acetyl-LDL alone. (B) Effects of 5 μM atorvastatin (ATV), 2 μM rosiglitazone (ROSI) and the combination of both agents on CD36 surface expression in THP-1 macrophages. Cells were simultaneously incubated with acetylated-LDL (150 μg/ml) in the presence or absence of the drugs under study for 24 h After treatments, CD36 expression was analyzed by incubation with a phycoeritrin (PE)-conjugated antibody against CD36 and determining cell fluorescence by flow cytometry. CD36 expression is represented as the percentage of mean fluorescence intensity. Data are the mean ± S.D. of three separate experiments run in triplicate. $^{**}P < 0.01$ respect control cells treated with acetyl-LDL alone. $^{*}P < 0.05$ vs. cells treated with rosiglitazone.

Regarding CD36, atorvastatin treatment showed a trend to reduce mRNA levels (21% reduction, non-statistically significant). On the contrary, incubation of cells with 2 μM rosiglitazone caused a marked increase (68%, $P < 0.05$) in CD36 mRNA expression. When the cells were exposed to atorvastatin and rosiglitazone, the inductive response elicited by rosiglitazone on CD36 mRNA levels was attenuated by a 34%, and statistical significance versus control cells was lost (Fig. 3A).

To investigate whether changes in CD36 mRNA levels were associated with modifications in CD36 protein, flow cytometry analysis for surface CD36 expression were performed. The results showed a similar trend (Fig. 3B), with rosiglitazone causing an increase of 2.2-fold ($P < 0.01$ versus cells treated with acetyl-LDL alone). The inductive response was reduced a 16% when atorvastatin was added ($P < 0.05$ versus rosiglitazone alone).

3.3. DiI-labeled oxLDL binding and uptake remain unaffected by rosiglitazone, atorvastatin and their combination

To determine whether the effects of the drugs on CD36 expression translated into changes in association of the

Table 2
Effect of atorvastatin, rosiglitazone and their combination on DiI-oxLDL binding and uptake by THP-1 macrophages

	Binding	Uptake
AcLDL	3.0 ± 0.9	27.1 ± 5.4
ATV	2.9 ± 1.2	26.7 ± 6.5
ROSI	2.4 ± 1.4	28.3 ± 8.3
ATV + ROSI	2.5 ± 0.5	26.3 ± 7.4

THP-1 macrophages were incubated with acetylated-LDL (150 μg/ml) in the presence or absence 5 μM atorvastatin (ATV), 2 μM rosiglitazone (ROSI) and the combination of both agents for 24 h. DiI-oxLDL uptake and binding were assessed by flow cytometry as described in Section 2. Data are expressed as mean fluorescence of the counted populations, and correspond to the mean ± S.D. of three or four independent experiments.

specific ligand of this receptor to macrophages, binding/uptake experiments using DiI-labeled oxLDL were performed. As it is shown in Table 2, despite the induction in CD36 elicited by rosiglitazone, oxLDL binding and uptake were not increased. Neither atorvastatin nor the combination of drugs significantly modified the binding and uptake of DiI-oxLDL.

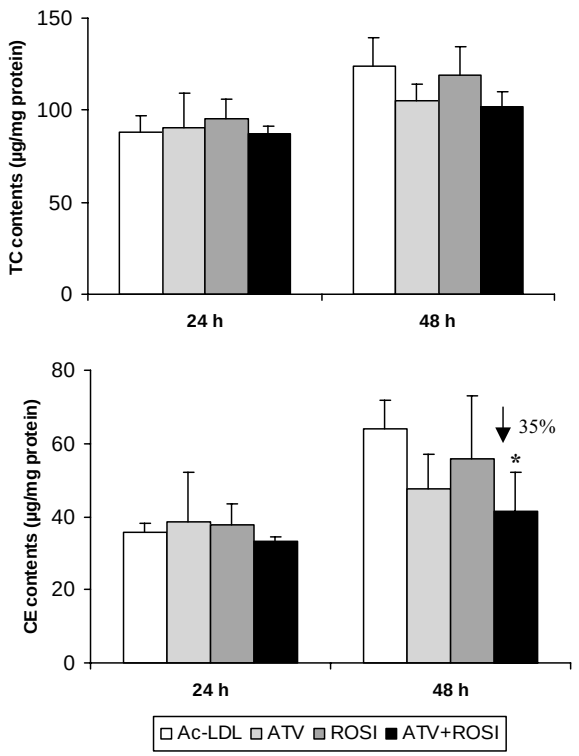

Fig. 4. Effect of 5 μM atorvastatin (ATV), 2 μM rosiglitazone (ROSI) or both agents combined on intracellular total (TC) and esterified cholesterol (EC) content. THP-1 macrophages were incubated simultaneously with the drug or drugs under study and 150 μg/ml acetyl-LDL for 24 h and 48 h. After washing, cellular lipid was extracted and total cholesterol (TC) and free cholesterol (FC) mass was determined. Cholesteryl ester (CE) content was calculated by subtracting FC from TC. Results are expressed as μg sterol/mg protein, and are the mean ± S.D. of three separate experiments using different batches of cells and lipoproteins, performed in duplicate wells. $^{*}P < 0.05$ respect control cells treated with acetyl-LDL alone.

3.4. Effect of treatments on intracellular cholesterol content

Differentiated THP-1 macrophages were treated simultaneously with acetyl-LDL and atorvastatin, rosiglitazone or the combination during 24 or 48 h, and the amount of intracellular free and total cholesterol were determined by gas chromatography. Cholesteryl ester (CE) mass was calculated by subtracting the content of free cholesterol from that of total cholesterol. As it is shown in Fig. 4, 24 h-treatments did not cause significant effects on intracellular cholesterol accumulation. When macrophages were treated for 48 h, atorvastatin alone or combined with rosiglitazone, reduced intracellular esterified cholesterol by 25 and 35%, respectively, although the decrease was only statistically significant for the combination treatment ($P < 0.05$). Rosiglitazone alone did not modify CE accumulation in any of the conditions assayed.

4. Discussion

The use of glitazones in diabetic patients, a population at a high risk of developing atherosclerosis, has been somewhat controversial since it was discovered that PPARγ agonists upregulated CD36, and therefore they could promote foam cell formation [5,31]. However, PPARγ ligands have been shown to reduce atherosclerotic lesions in animal models [32–34], and it has been recently reported that ciglitazone may reduce CE accumulation in THP-1 macrophages [8]. In contrast with these results, other studies show either no effect [35] or even an increase [11] in macrophage cholesterol content after treatment with PPARγ ligands. Moreover, despite the favourable effects exerted by the combination of a glitazone and a statin on plasma lipid levels in patients with type 2 diabetes [6], the effects of this combination on foam cell formation in macrophages have never been directly examined. For this purpose, we studied the effects of rosiglitazone and atorvastatin, alone and combined, on the expression of CD36 and other genes involved in cholesterol entry and efflux in THP-1 human macrophages, and determined if the changes induced by the treatments translated into modifications in intracellular cholesterol accumulation. We used a protocol in which differentiated THP-1 macrophages were exposed simultaneously to acetyl-LDL and the drug or drugs under study, in order to assess their effects during the process of lipid-loading of the macrophage [26,36].

Under these conditions, none of the treatments significantly modified the mRNA levels of genes involved in cholesterol efflux from these cells (Table 1). Other authors found an increase [13] or a decrease [37] in apo E mRNA levels in macrophages treated with statins. Castilho et al. [37] attributed the reduction in apo E to the statin-induced decrease in intracellular cholesterol content. Therefore,

besides differences in culture conditions, the discrepancies with our results may be attributed to the fact that we did not detect a reduction in macrophage cholesterol after treatment with atorvastatin for only 24 h. Our findings also contrast with those of others who found that PPARγ agonists increase ABCA1 gene expression in macrophages through a transcriptional cascade mediated by LXRα [10,35]. However, some recent studies from our group [38] and others [9,39] have also failed to detect an inductive effect of rosiglitazone on ABCA1 mRNA. We can exclude that under the conditions applied in this study there is a lack of efficacy of rosiglitazone as PPARγ ligand, as it is capable of stimulating CD36 gene expression (Fig. 3), and it has been reported that comparable concentrations of PPARγ agonists induce ABCA1 and CD36 mRNA in cultured macrophages [8,40]. Since it has been described that in some tissues there is not a good correlation between the abundance of ABCA1 mRNA and protein [41], we also examined the levels of ABCA1 protein by immunoblotting. Both rosiglitazone and the combination of rosiglitazone and atorvastatin caused a marked increase in the amount of ABCA1 protein. Similarly, while none of the treatments significantly affected the mRNA of CLA-1, the human homologue of SR-BI, we found that CLA-1 protein expression was upregulated after rosiglitazone treatment, alone or combined with atorvastatin. This is in accordance with a previous report showing that treatment of 12-day-old human monocyte-derived macrophages with rosiglitazone increased by three-fold the amount of CLA-1 protein [42].

CD36 mRNA levels were strongly induced by rosiglitazone exposure, while atorvastatin caused a slight reduction. These effects are in accordance with other studies in macrophages treated with PPARγ agonists [5] or statins [43,44]. Probably as a result of the divergent responses elicited by rosiglitazone and atorvastatin on CD36 mRNA, when THP-1 macrophages were treated with a combination of both drugs, the inductive response was halved. Confirming this finding, the increase in CD36 cell surface expression induced by rosiglitazone was also significantly attenuated when cells were incubated with the combination of rosiglitazone and atorvastatin.

Taking these results together, we hypothesized that while similar amounts of cholesterol would be exported via CLA-1 or ABCA1 from macrophages treated with the combination rosiglitazone-atorvastatin, compared to rosiglitazone alone, less cholesterol would be taken up by these cells, as the increase in CD36 expression was significantly attenuated. Since CD36 preferentially scavenges oxLDL [8,15], we performed binding experiments with this ligand to confirm this hypothesis. However, none of the treatments significantly affected the binding of DiI-labeled oxLDL to THP-1 macrophages. Conflicting results on the influence of PPARγ-induced CD36 activation on the binding and uptake of modified lipoproteins have been reported. Thus, despite a marked induction in CD36 expression, PPARγ and RXR ligands only modestly enhanced oxLDL uptake in THP-1 cells [5,8], while in macrophages derived from embryonic stem (ES) cells, [^{125}I] oxLDL uptake was unaffected by troglitazone [4]. Moreover, oxLDL uptake may not be considered as a good predictor of macrophage CE accumulation.

The expression of CD36, ABCA1, ABCG1, and CLA-1 is subjected to sterol-dependent regulation in macrophages [16,45,46]. This raises the possibility that the effects on gene expression reported in the present study were a consequence of differences in intracellular cholesterol status induced by the drugs. We can exclude this possibility, as gene expression is already affected after 24 h of treatment, while macrophage cholesterol levels are still not modified (Fig. 4).

Rosiglitazone alone did not affect intracellular total or esterified cholesterol levels even when treatments were extended to 48 h. Our results differ from those of Argmann et al. [8], who demonstrated that ciglitazone significantly reduced CE accumulation induced by atherogenic lipoproteins, but are in agreement with those reported by Chinetti et al. [35] in THP-1 and human primary macrophages treated with rosiglitazone. The disparities can be attributed to the use of different glitazones, different doses or length of exposure to the ligands.

In contrast with the results obtained with rosiglitazone alone, we found that when the PPARγ ligand was combined with atorvastatin there was net depletion (35%) in CE content in macrophages. Under our experimental conditions, this reduction does not seem to be a consequence of the modifications on the expression of SR-AI, CD36, ABCA1 or CLA-1. Atorvastatin alone also reduces by 25% intracellular CE accumulation, although statistical significance is not achieved. The decrease in CE induced by HMG-CoA reductase inhibitors in lipid loaded macrophages has been previously described [12,13,26]. This effect is mostly related to a reduction in CE formation, which has been attributed to an indirect inhibition of acyl-CoA:cholesterol acyltransferase activity by limitation of substrate availability [47]. Cignarella et al. [13] proposed that the drop in intracellular CE contents in macrophages incubated with lovastatin may be related to an impairment in the delivery of free cholesterol to the plasma membrane. Whatever the mechanism involved, the inhibition of CE formation exerted by atorvastatin may be responsible, at least in part, for the decrease in cholesterol accumulation found after treatment of macrophages with the combination of rosiglitazone and atorvastatin.

Our observations imply that, in addition to the beneficial effects induced by each agent on glycemic control and plasma lipid parameters [6], the combination of rosiglitazone and atorvastatin may be directly antiatherogenic. It should be stressed that even under experimental conditions where rosiglitazone alone does not reduce intracellular macrophage sterol levels, the combination with atorvastatin confers the ability to prevent CE accumulation. This

effect, that can contribute to reduce foam cell formation, adds interest to the clinical use of the combination therapy in type 2 diabetic patients, who are at a high risk of developing atherosclerotic lesions.

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