Ritonavir Increases CD36, ABCA1 and CYP27 Expression in THP-1 Macrophages

JORDI POU,* ALBA REBOLLO,* NÚRIA ROGLANS,* ROSA M. SÁNCHEZ,* MANUEL VÁZQUEZ-CARRERA,* JUAN C. LAGUNA,* JUAN PEDRO-BOTET,† AND MARTA ALEGRET*,[1]

Pharmacology Department, Faculty of Pharmacy and Biomedicine Institute (IBUB), University of Barcelona, and Ciber Diabetes y Enfermedades Metabólicas asociadas (CIBERDEM), Instituto de Salud Carlos III, Spain and †Department of Medicine, Hospital del Mar, Universidad Autónoma de Barcelona, Spain

Ritonavir, a protease inhibitor used in combination antiretroviral therapy for HIV-1 infection, is associated with an increased risk of premature atherosclerosis. The aim of the present study was to assess the effects of ritonavir, in the absence of added lipoproteins, on the expression of genes that control cholesterol trafficking in human monocytes/macrophages. ***Design:*** **THP-1 cells were used to study the effects of ritonavir on the expression of CD36, ATP binding cassette transporters A1 (ABCA1) and G1 (ABCG1), scavenger receptor B class I (SR-BI), caveolin-1 and sterol 27-hydroxylase (CYP27). Exposure to ritonavir (2.5 μg/ml) increased CD36 protein (28%, $P < 0.05$) and mRNA (38%, $P < 0.05$) in differentiated THP-1 macrophages, but not in undifferentiated monocytes. This effect was not related to the increase in PPARγ expression (51%, $P < 0.05$) caused by ritonavir. Ritonavir also reduced SR-BI protein levels (46%, $P < 0.05$) and increased CYP27 (43%, $P < 0.05$) and ABCA1 (49%, $P < 0.05$) mRNA expression. Liver X receptor α (LXRα) mRNA, protein and binding activity were also increased by ritonavir treatment.** ***Conclusions:*** **We propose that ritonavir induces ABCA1 expression in THP-1 macrophages through LXRα. The increase in ABCA1 and other cholesterol efflux mediators, such as CYP27, may compensate CD36 induction. Therefore, we suggest that the net effect of ritonavir on macrophages in the absence of lipoproteins is not clearly pro-atherogenic.** Exp Biol Med 233:1572–1582, 2008

Key words: ritonavir; macrophage; CD36; ABCA1; sterol 27-hydroxylase

[1] To whom correspondence should be addressed at Diagonal 643, Barcelona 08028, Spain. E-mail: alegret@ub.edu

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Combination antiretroviral therapy (CART) has dramatically changed the natural history of human immunodeficiency virus (HIV) infection, leading to a significant decrease in morbidity and mortality and a notable prolongation of life expectancy (1, 2). However, the potential for maintaining HIV-infected patients on treatment for decades may be limited by the various adverse effects observed in patients on CART such as dyslipidemia, fat redistribution, insulin resistance and premature atherosclerosis (3–6), particularly when therapy contains protease inhibitors (PIs) (7–11). This fact has raised the concern that the HIV-infected population may be at increased risk for cardiovascular disease in the long term, as described in two large prospective studies (12, 13). At present, the relationship between PIs and atherosclerosis in HIV-infected patients remains unclear, and the mechanisms underlying this association remain unknown. In addition to their proatherogenic metabolic abnormalities, direct effects of PIs on different cell types involved in atherogenesis have been reported (14–19).

One of the earliest events in atherosclerosis development is macrophage lipid loading, which leads to foam cell and fatty streak formation. This process is mediated by a group of scavenger receptors expressed on these cells, particularly SR-A and CD36 (20). An early and marked down-regulation of CD36 on monocytes induced by PIs has been proposed as a possible cause of insulin resistance and dyslipidemia in HIV-infected patients (21). In contrast, other authors described a PI-driven up-regulation of CD36 leading to accumulation of sterols in macrophages, thereby implying a proatherogenic effect of PIs (19, 22, 23). In addition to the opposing findings of these studies, which could be explained at least in part by differences in experimental design, data on other molecules involved in macrophage cholesterol efflux, such as ATP-binding cassette transporters ABCA1 (24) and ABCG1 (25), scavenger receptor class B type I (SR-BI) (26), sterol 27-

Table 1. Primers Used for the Polymerase Chain Reaction (PCR)[a]

Gene	Gene sequence of primers (5′-3′)	Product length (bp)	Number of cycles	Linear range
ABCA1	forward: GGAGGCAATGGCACTGAGGAA reverse: CCTGCCTTGTGGCTGGAGTGT	181	18	18–23
ABCG1	forward: CCATGATGGTGTCGGCACATC reverse: GCTGGTGGGCTCATCGAAGAA	206	22	20–25
Caveolin-1	forward: ACAAGCCCAACAACAAGGCCA reverse: GAGGGCAGACAGCAAGCGGTA	245	25	20–25
CD36	forward: CTGTGACCGGAACTGTGGGCT reverse: GAAGATGGCACCATTGGGCTG	361	18	18–23
CYP27	forward: GCCATGGGCAGCCTGCCTGA reverse: CTTGCGAGGAGTAGCTGCATC	502	23	20–25
SR-BI	forward: ACGACACCGTGTCCTTCCTCG reverse: CGGGCTGTAGAACTCCAGCGA	509	19	18–23
PPARγ	forward: CATTCTGGCCCACCAACTTTGG reverse: TGGAGATGCAGGCTCCACTTTG	229	20	19–23
LXRα	forward: AGCCCCCTTCAGAACCACAG reverse: AGGACACACTCCTCCCGCATG	295	23	20–25
GAPDH	forward: CAGTCCATGCCATCACTGCCA reverse: AGGTGGAGGAGTGGGTGTCGC	302	18	18–23

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

hydroxylase (CYP27) (27) and caveolin-1 (28) are lacking. Thus, the aim of the present study was to assess the effects of ritonavir, in the absence of added lipoproteins, on the expression of genes that control cholesterol trafficking in THP-1 monocytes/macrophages.

Materials and Methods

Cell culture reagents were from Gibco, Invitrogen Corporation, with the exception of fetal bovine serum, purchased from Sigma-Aldrich. Trizol and RT-PCR reagents were from Invitrogen Corporation, 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 SREBP-1 from Santa Cruz, and anti-β-actin antibody was from Sigma-Aldrich. Other general chemicals were obtained from commercial sources and were of analytical grade.

Cell Culture. THP-1 monocytoid cells were obtained from the European Collection of Cell Cultures (ECACC) and kept in RPMI 1640 medium 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. Monocytes were exposed to complete medium supplemented with 50 ng/ml PMA for 24 h and then incubated with different ritonavir concentrations (0.05–2.5 μg/ml) for 24 h. In some cases, non-differentiated THP-1 monocytes were also used. Drug solutions were prepared in ethanol, with the final ethanol concentration being 0.1%.

Cytotoxicity Assays. Release of lactate dehydrogenase (LDH) into the culture medium was evaluated after 24 h of incubation with various concentrations of ritonavir (0.05–10 μg/ml) by using the LDH assay kit (Roche Molecular Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. The spectrophotometric absorbance of the colored formazan was determined at 490 nm wavelength and 690 nm reference wavelength. LDH release was measured as percentage of LDH leakage:

$$\%LDH\ release = [(A_S - A_L)/(A_H - A_L)] \times 100,$$

where, A_S = absorbance of the treated sample, A_L = absorbance of control sample (low control), and A_H = absorbance of samples treated with 0.3% triton-X100 (a control for maximum LDH release (high control). Results of three independent experiments (by duplicate) are expressed as average percentages ± standard deviation.

Cell viability was also determined by measuring the ability of THP-1 cells to reduce MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and form a formazan product, which can be quantified spectrophotometrically at 570 nm (29). Results are expressed as % versus the absorbance of control samples, and are the mean ± standard deviation of three independent experiments performed in duplicate.

RNA Preparation and Analysis. Specific mRNA levels were assessed by reverse transcription-polymerase chain reaction (RT-PCR), as previously described (30). The sequences of the forward and reverse primers used for PCR amplification, length of the PCR product, number of cycles used in the PCR and linear range for the amplification are given in Table 1. PCR was performed in an MJ Research Thermocycler equipped with a peltier system and temperature probe. Results for specific mRNA expression were normalized to the expression of the control gene (*gadph*), and expressed as expressed as the change in gene expression in percentage compared with the control situation (100%).

Western Blot Analysis. Protein extracts from con-

Table 2. Effects of Ritonavir on Cell Viability in THP-1 Macrophages[a]

Control	0.05 μg/ml	0.1 μg/ml	0.25 μg/ml	0.5 μg/ml	1 μg/ml	2.5 μg/ml	5 μg/ml	10 μg/ml
LDH leakage assay (% cytotoxicity)								
8.6 ± 3.1	8.9 ± 3.7	7.4 ± 3.0	8.5 ± 4.5	7.6 ± 6.0	11.2 ± 5.1	15.8 ± 6.0	27.1 ± 8.8**	45.4 ± 4.1***
MTT assay (% cell viability)								
100	101 ± 7	95 ± 13	90 ± 13	95 ± 15	82 ± 22	84 ± 24	nd	nd

[a] LDH release to the medium was determined on THP-1 macrophages treated with vehicle alone (control) or with 0.05–10 μg/ml ritonavir for 24 h, and results are expressed as percentage of cytotoxicity as described in the Methods section. The MTT assay was also performed on THP-1 macrophages treated with vehicle alone (control) or with 0.05–2.5 μg/ml ritonavir for 24 h, and results are expressed as percentage of viability compared to vehicle-treated cells (control group), which was set at 100%.
** $P < 0.01$; *** $P < 0.001$ (ANOVA test).

trol and treated cells were obtained as previously described (31), and membrane and crude nuclear fraction were prepared from THP-1 macrophages according to Matsuoka et al. (32) with several modifications. Briefly, cells were homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing 10 μM phenylmethylsulfonylfluoride, 2 μg/ml aprotinin, 1 μg/ml leupeptin and 1 mM sodium orthovanadate, and centrifuged at 21,000 *g* at 4°C for 20 min. The supernatant was used as the membrane fraction and the pellets were resuspended in RIPA buffer (Sigma), kept on ice for 30 min and centrifuged at 12,000 *g* at 4°C for 15 min. The supernatant was then used as the nuclear fraction. Forty μg of protein were subjected to 12% SDS-polyacrylamide gel electrophoresis and then transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore). The membranes were blocked for 1 h at room temperature in phosphate saline buffer containing 5% non-fat dry milk, and proteins were immunologically detected using rabbit polyclonal antibodies against SR-BI (dilution 1:500, 1.5 h at room temperature), ABCA1 (dilution 1:500, overnight at 4°C) and SREBP-1 (1:200 dilution, overnight at 4°C) or a goat polyclonal antibody against LXRα (1:100 dilution, overnight at 4°C).

After several washes, blots were incubated with an appropriate secondary antibody, and detection was achieved using the enhanced chemiluminescence (ECL) detection system (Amersham Bioscience). Blots were also incubated with a monoclonal antibody raised against β-actin used as a control of equal loading of protein. Size of detected proteins was estimated using protein molecular-mass standards (BioRad). Chemiluminescence was detected using a Chemidoc XRS (BioRad), and densitometric analysis was performed by Quantity One® software. Each band was quantified and normalized to β-actin signal. Protein levels are expressed as percentage of controls (100%).

Electrophoretic Mobility Shift Assays (EMSA). The DNA sequence of the double-stranded oligonucleotide used (LXR response element from ABCA1 promoter) was 5′-TGGGCTTTGACCGATAGTAACCTCTGCGC-3′ (33). Oligonucleotides were labeled in the following reaction: 2 μl of oligonucleotide (100 ng/μl), 2 μl of 5 × kinase buffer, 5 U of T4 polynucleotide kinase (Invitrogen Life Technologies), and 3 μl of [γ-^{32}P] ATP (3000 Ci/mmol at 10 mCi/ml, Amersham Biosciences) incubated at 37 °C for 2 h. The reaction was stopped by adding 90 μl of TE buffer (10 mM Tris-HCl (pH 7.4) and 1 mM EDTA). To separate the labeled probe from the unbound ATP, the reaction mixture was eluted in a Nick column (Pharmacia) according to the manufacturer's instructions. Three micrograms of crude nuclear proteins was incubated for 10 min on ice in binding buffer (10 mM Tris-HCl (pH 8.0), 25 mM KCl, 0.5 mM DTT, 0.1 mM EDTA (pH 8.0), 5% glycerol, 5 mg/ml BSA and 50 μg/ml poly(dI-dC)), in a final volume of 10 μl. Labeled probe (approximately 70,000 cpm) was added and the reaction was incubated for 20 min at room temperature. Where indicated, non-radioactive specific competitor oligonucleotide was added before the addition of labeled probe and incubated for 15 min on ice. Radioactive bands were quantified by video-densitometric scanning.

Flow Cytometry for Surface Expression of CD36. Immunofluorescent flow cytometry was carried out using a FITC-conjugated mouse monoclonal antibody against CD36 (Serotec), as previously described (34), and analyzed with a flow cytometer (Epics XL).

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 GraphPad InStat computer program was used for the calculations.

Results

Ritonavir Increased CD36 and ABCA1 and Reduced SR-BI Protein Levels. Since ritonavir concentrations reported for in vitro studies with macrophages vary widely, we first treated THP-1 cells with increasing concentrations (from 0.05 to 10 μg/ml) and evaluated cytotoxicity by two methods: LDH leakage and MTT. Both assays showed that cytotoxicity was marked at concentrations higher than 2.5 μg/ml (Table 2). THP-1 monocytes and macrophages were then treated with ritonavir (0.05–2.5 μg/ml) and protein levels of CD36, ABCA1 and SR-B1 were determined. At the highest concentration tested,

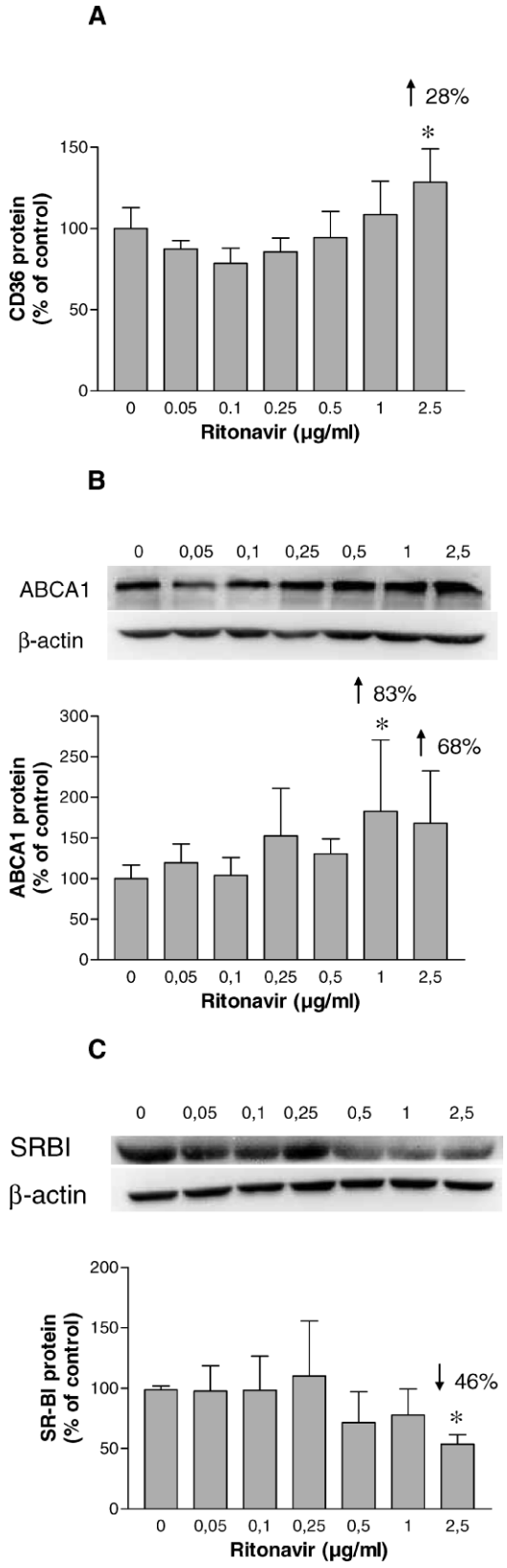

Figure 1. Ritonavir increases CD36 and ABCA1 protein levels. THP-1 macrophages were incubated with various concentrations (0.05–2.5 μg/ml) of ritonavir during 24 h. **A**: Analysis of CD36 surface protein expression by flow cytometry, using a FITC-conjugated antibody against CD36. Data are expressed as the percentage of mean fluorescence intensity ± SD of 3 independent experiments run in duplicate. **B, C:** Whole protein extracts (40 μg) were resolved in 12% SDS-polyacrylamide gel. The blots were analysed with anti-ABCA1 (**B**) and anti-SR-BI (**C**) antibodies. Representative autoradiograms are shown. Bands were quantified, normalized to β-actin signal and the change in protein expression was calculated in comparison with the control situation (100%). Data are expressed as mean ± standard deviation (SD) of 3–4 independent experiments run in duplicate. * $P < 0.05$ versus control cells (ANOVA test).

ritonavir increased CD36 cell surface expression by 28% (Fig. 1A, $P < 0.05$), while it was ineffective on non-differentiated THP-1 monocytes (data not shown). Ritonavir treatment also increased macrophage ABCA-1 protein levels (Fig. 1B, 83% increase at 1 μg/ml, $P < 0.05$), while SR-BI protein expression was significantly reduced (Fig. 1C, 46% decrease at 2.5 μg/ml, $P < 0.05$).

Ritonavir Increased CD36 and ABCA1 mRNA Expression. THP-1 macrophages were treated with ritonavir (1 and 2.5 μg/ml) and RT-PCR analysis was performed to ascertain whether changes in protein levels were associated with modifications in the corresponding mRNA. Results showed that ritonavir treatment increased CD36 mRNA by 38% (Fig. 2A, $P < 0.05$) and ABCA1 mRNA (32% and 49% increase at 1 and 2.5 μg/ml, respectively, $P < 0.05$, Fig. 2B), while SR-BI mRNA levels were not significantly altered (Fig. 2C). In addition, the mRNA expression of two other molecules involved in cholesterol efflux, ABCG1 and caveolin-1 (cav-1) was analyzed. As shown in Figure 2D–E, the expression of both genes was unaffected by exposure to ritonavir.

PPARγ Expression Was Increased in Ritonavir-Treated Macrophages. Treatment with 2.5 μg/ml ritonavir raised PPARγ mRNA levels by 51% ($P < 0.05$) (Fig. 3A). It has been suggested that the effects of PIs on PPARγ could be mediated by activated SREBP accumulation in the macrophage nucleus. Immunoblot analysis of the membrane fraction of cell homogenates revealed a band at ~125 kDa corresponding to the precursor form (pSREBP-1), and a ~67-kDa protein in the nuclear fraction corresponding to active nuclear form (nSREBP-1). Ritonavir treatment (2.5 μg/ml, 24 h) did not change pSREBP-1 levels compared with control, whereas nSREBP-1 abundance was significantly increased (Fig. 3B).

To further explore the involvement of PPARγ in the observed ritonavir effects, we treated the cells with ritonavir alone or combined with a PPARγ antagonist (GW9662, 20 μM) or a PPARγ agonist (rosiglitazone, 2 μM) and determined the expression levels of CD36, ABCA1 and sterol 27-hydroxylase (CYP27), another protein related to cholesterol efflux known to be induced by PPARγ agonists. Our results showed that pre-treatment with GW9662 did not block the increase in CD36 or ABCA1 mRNA levels (Fig. 4A–B). In fact, ABCA1 induction was significantly greater when GW9662 was present (116% vs 47% increase in the absence of GW9662, $P < 0.001$). Interestingly, 2.5 μg/ml ritonavir increased the mRNA levels of CYP27 by 43% (*P*

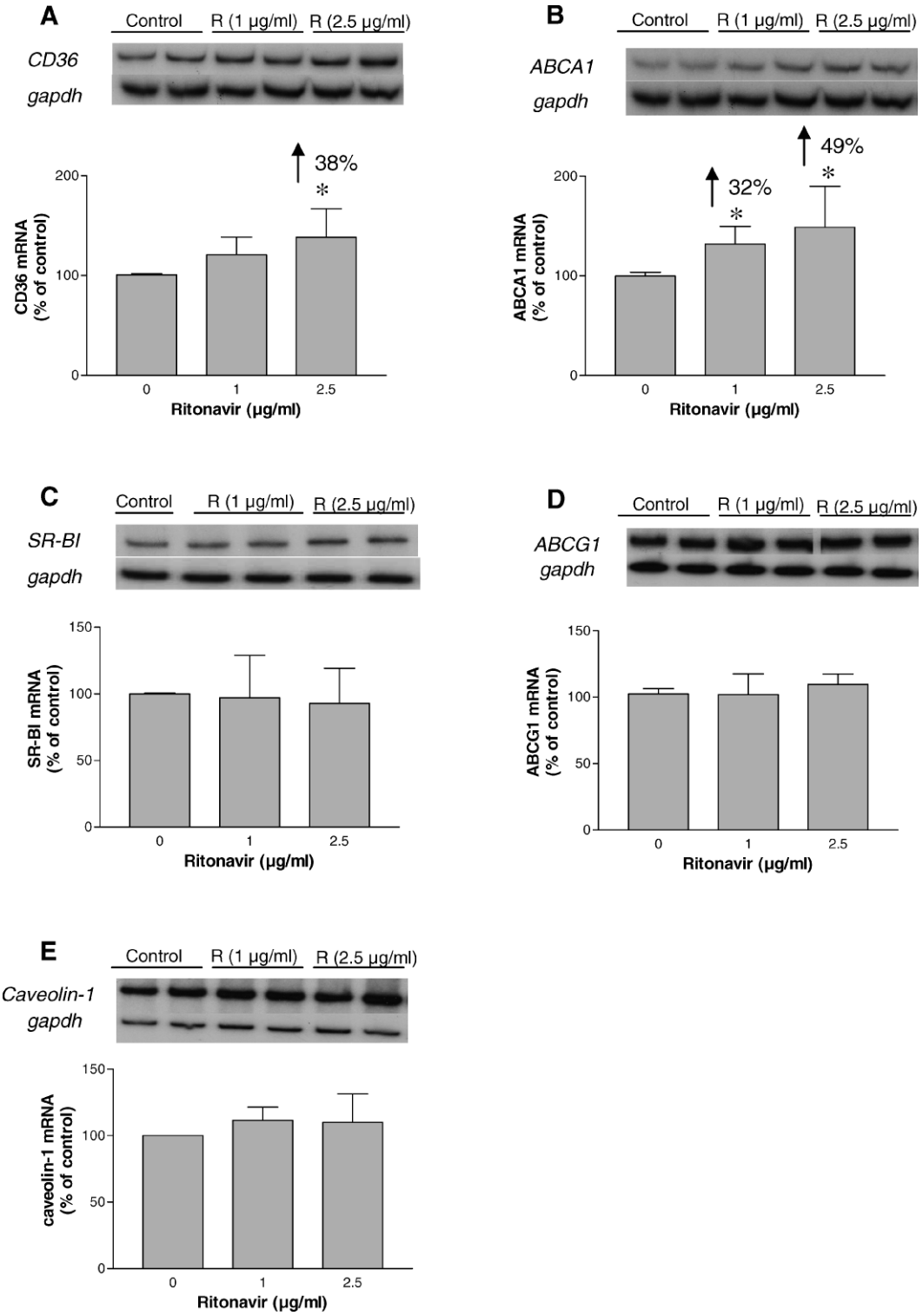

Figure 2. Ritonavir increases CD36 and ABCA1 mRNA expression. THP-1 macrophages were incubated in the absence (CT) or in the presence of ritonavir (1 and 2.5 µg/ml) during 24 h. Analysis of CD36 (**A**), ABCA1 (**B**), SR-BI (**C**), ABCG1 (**D**) and caveolin-1 (**E**) mRNA levels. 0.5 µg of total RNA was analyzed by RT-PCR. Representative autoradiograms are shown. Bands were quantified, normalized to the gapdh signal and the change in mRNA levels was calculated in comparison with the control situation (100%). Data are expressed as mean ± standard deviation (SD) of 3 independent experiments run in duplicate. * $P < 0.05$ versus corresponding control cells (ANOVA test).

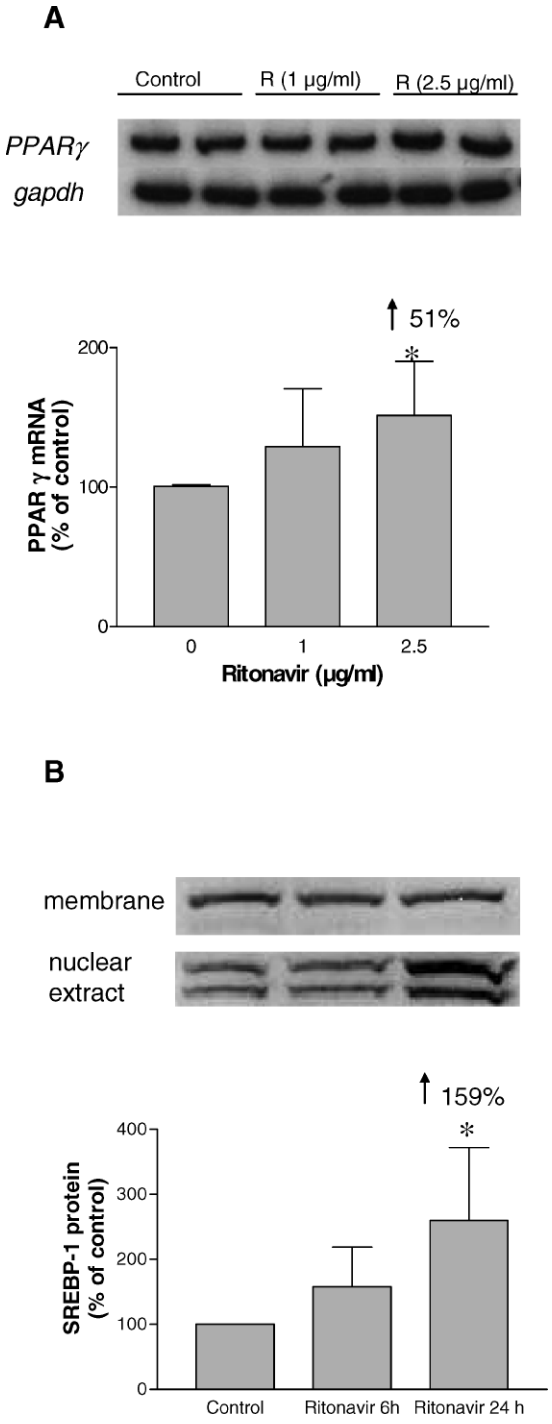

Figure 3. PPARγ expression and mature SREBP-1 are increased in ritonavir-treated macrophages. **A**: Analysis of PPARγ mRNA levels in THP-1 macrophages exposed to ritonavir (1 and 2.5 µg/ml) during 24 h. 0.5 µg of total RNA were analyzed by RT-PCR. A representative autoradiogram is shown. Bands were quantified, normalized to the gapdh signal and the change in mRNA levels was calculated in comparison with the control situation (100%). Data are expressed as mean ± SD of 3 independent experiments. * $P < 0.05$ compared with control cells (ANOVA test). **B**: Immunoblot analysis of the SREBP-1 precursor form was performed in the membrane fraction, whereas analysis of the active, nuclear form of SREBP-1 was performed in the nuclear fraction. A representative autoradiogram and quantifications are shown. Data are expressed as the band intensity ratio of mature versus precursor form relative to control, and correspond to the mean values ± SD of 5 independent experiments. * $P < 0.05$ compared with control cells (ANOVA test).

< 0.05) and, in this case pre-treatment with GW9662 abolished the stimulatory effect, thereby lowering mRNA levels by nearly 40% ($P < 0.001$) (Fig. 4C). On the other hand, the effect of ritonavir on CD36, ABCA1 or CYP27 was not modified by the addition of rosiglitazone.

ABCA1 Increase Was Related to LXR but Not to the cAMP-PKA Pathway. We were interested in studying the mechanisms by which ritonavir induces ABCA1 expression in macrophages. It has been described that liver X receptor (LXR) mRNA is transcriptionally controlled by PPARγ agonists, leading to ABCA1 induction (35). Therefore, we analysed the LXR mRNA levels in macrophages treated with ritonavir, and found that ritonavir (2.5 µg/ml) significantly increased the mRNA expression of LXRα by 36% ($P < 0.01$) (Fig. 5A). Moreover, protein levels of LXRα were also increased in the nuclear fraction of ritonavir-treated THP-1 cells (Fig. 5B). Further, we examined whether ritonavir could increase the binding of LXR to a LXR response element (LXRE) in the ABCA1 promoter. The incubation of macrophage nuclear extracts with a LXRE oligonucleotide probe yielded a single, specific band that disappeared in the presence of increasing amounts of unlabeled probe (Fig. 5C). Nuclear extracts from macrophages treated with ritonavir (2.5 µg/ml, 24 h) showed a 61% increase ($P < 0.05$) in the intensity of the specific band corresponding to the binding of LXR to an ABCA1 specific LXRE (Fig. 5D).

ABCA1 expression is also regulated independently of the LXR system by cyclic AMP via a protein kinase A (PKA)-mediated process. However, the pharmacological blockade of the PKA pathway using H89, a specific PKA inhibitor, did not reverse ABCA1 induction driven by ritonavir (Fig. 5E). Taken together, these results suggest that ritonavir stimulates ABCA1 expression through LXRα in a cAMP-PKA-independent manner.

Discussion

The present study demonstrates that ritonavir has a significant impact on the expression of master regulators of cholesterol trafficking in human macrophages by lowering SR-BI and increasing CD36, ABCA1 and CYP27 expression. These findings may be clinically significant, since the effective ritonavir concentrations used in the present study were within the range of pharmacological steady-state plasma concentrations observed after a 600 mg/12 h dose in human subjects (23). In contrast to other studies, the experiments were performed in the absence of any added lipoprotein to better ascertain the ritonavir-specific effects. In this respect, it is well known that some components of modified lipoproteins and cholesterol loading in themselves induce changes in the expression of these cholesterol-related genes (31); thus, the observed ritonavir effects may have been influenced by co-incubation with these lipoproteins.

Several studies demonstrated that ritonavir and other PIs alter the expression of CD36, but discrepancies existed

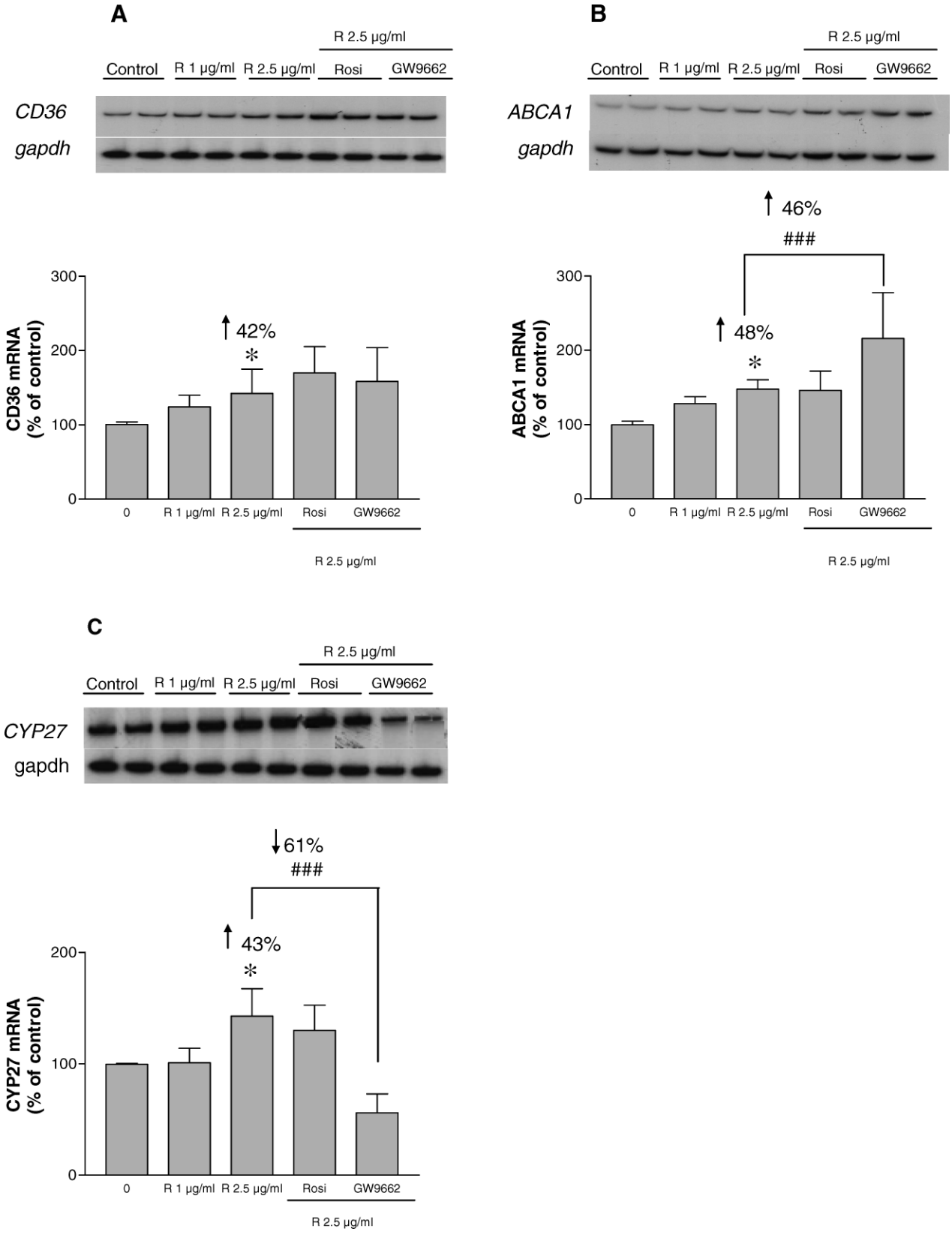

Figure 4. A PPARγ antagonist (GW9662) does not affect CD36 or ABCA1 induction, but reverses the increase in CYP27 expression caused by ritonavir. Analysis of CD36 (**A**) ABCA1 **(B)** and CYP27 **(C)** mRNA levels in THP-1 macrophages incubated with ritonavir 1 µg/ml and ritonavir 2.5 µg/ml (alone or combined with rosiglitazone (2 µM) or GW 9662 (20 µM)) during 24 h. 0.5 µg of total RNA was analyzed by RT-PCR. Representative autoradiograms are shown. Bands were quantified, normalized to the gapdh signal and the change in mRNA levels was calculated in comparison with the control situation (100%). Data are expressed as mean ± SD of 3 independent experiments run in duplicate. * $P < 0.05$ versus corresponding control cells. ### $P < 0.001$ vs cells treated with 2.5 µg/ml ritonavir (ANOVA test).

among these studies; some showed a decrease (21) and others an increase in CD36 (22, 23). It was speculated that PIs caused differential effects depending on the differentiation status of the monocyte-macrophage (36). Our results also point to a different effect of ritonavir on THP-1 monocytes where CD36 surface levels were unaffected by the drug treatment, and THP-1 differentiated macrophages where an increase was observed in both CD36 surface expression and mRNA levels (Figs. 1A and 2A). One of the mechanisms proposed for CD36 induction by PIs involves changes in the expression or activity of PPARγ (21, 22). However, co-incubation with a PPARγ antagonist (GW9662) did not inhibit ritonavir-mediated induction of CD36, indicating that this effect was independent of PPARγ signaling.

Regarding the mechanism underlying the increase in PPARγ mRNA by ritonavir, it has been reported that PPARγ expression can be controlled by the SREBP family of transcription factors (37). It has been proposed that PI may cause the accumulation of activated SREBPs in the macrophage nucleus (19). In agreement with this hypothesis, we observed an increase in mature SREBP-1 levels in nuclear extracts of THP-1 macrophages treated with ritonavir 2.5 μg/ml for 24 h (Fig. 3B). These results suggest that the increase in mature SREBP-1 caused by ritonavir may mediate the observed PPARγ induction. It is noteworthy that, in contrast to Zhou *et al.* (19), we did not observe an increase in SREBP-1 precursor form, which points to an effect of ritonavir on SREBP-1 processing rather than on SREBP-1 transcription.

Our results also show that ABCA1 is induced by exposure to ritonavir. Zhou *et al.* (19) observed a similar trend in murine macrophages treated with ritonavir 15 μM, but the increase did not reach statistical significance. In contrast, ABCA1 expression was not modified in ritonavir-treated THP-1-derived foam cells (38). In foam cells, the intracellular accumulation of sterols and oxysterols could activate LXR and result in higher basal ABCA1 levels, which ritonavir would not increase further. The absence of cholesterol loading in our cell model may have facilitated ABCA1 induction by ritonavir. It has been shown that macrophage ABCA1 expression is controlled by a PPARγ-LXR pathway (35). However, pre-treatment with the PPARγ antagonist GW9662 did not reverse the inductive effect of ritonavir on ABCA1, which was even greater than that observed with ritonavir alone (Fig. 4A). This effect could have been mediated by GW9662 working as a partial PPARα agonist, as other authors suggested previously (39, 40). Chawla *et al.* (2001) described that PPARγ agonists induce LXRα mRNA expression, leading to ABCA1 upregulation. Although PPARγ does not seem to play a major role in ABCA1 induction by ritonavir, this compound could act directly by increasing LXR expression. Accordingly, we observed a significant increase in LXRα mRNA after ritonavir exposure (Fig. 5A), which suggests that the upregulation of ABCA1 by ritonavir is independent of PPARγ and could be directly mediated through LXR. To gain further insight into the mechanism regulating ABCA1 expression by LXR, we performed EMSA experiments using as the probe a double stranded oligonucleotide that contained the LXRE identified in the ABCA1 promoter. This probe originated single band upon incubation with THP-1 nuclear extracts, and the observed binding was increased when THP-1 macrophages were treated with ritonavir. This suggests that the effect of ritonavir on ABCA1 expression may be mediated by transcription activation upon binding of LXR-RXR heterodimers to the LXRE in the promoter of the ABCA1 gene. However, the effect of ritonavir on LXRα expression may not be strong enough to induce ABCG1, another LXR target gene (41).

Interestingly, ritonavir treatment significantly increased mRNA levels of CYP27 (Fig. 4B), another protein involved in cholesterol efflux from macrophages (27, 42). Our results show that co-incubation with a PPARγ antagonist abolishes the induction caused by ritonavir, which indicates that in this case the effect is PPARγ-dependent. The CYP27 product 27-hydroxycholesterol is the most likely endogenous ligand for LXRα in macrophages, and overexpression of CYP27 has been shown to activate the LXR/RXR system (43). Moreover, treatment of cells with 27-hydroxycholesterol increases ABCA1 gene expression to a greater extent than ABCG1 (43). Therefore, it is tempting to speculate that the increase in 27-hydroxycholesterol due to CYP27 stimulation activates LXR enough to induce ABCA1, but not ABCG1, expression in THP-1 macrophages.

Taken together, our results suggest that the increase in CD36 expression, which is regarded as pro-atherogenic, may be compensated by an increase in the expression of genes involved in cholesterol efflux, ABCA1 and CYP27. The decrease in SR-BI protein levels caused by ritonavir would not impair this process, as it has recently been reported that SR-BI does not promote reverse cholesterol transport *in vivo* (44). Earlier reports in human macrophages exposed to atherogenic lipoproteins (22, 38) showed that ritonavir effects were clearly pro-atherogenic. Thus, CD36 overexpression was observed in THP-1 macrophages and peripheral blood mononuclear cells after treatment with ritonavir in combination with aggregated LDL (22, 45); on the other hand, ritonavir reduced SR-B1 and caveolin-1 expression in THP-1 monocytes and peripheral blood mononuclear cells preincubated with acetylated-LDL to form foam cells; this led to a reduction in cholesterol efflux, despite ABCA1, ABCG1 and CYP27 expression was not modified (38). The accumulation of cholesterol due to increased influx and decreased efflux was suggested to be at the basis of the higher atherosclerotic risk in patients treated with PI. In contrast, our findings suggest that ritonavir in the absence of hyperlipidemia would not exert pro-atherogenic effects on vascular cells such as macrophages. However, we did not measure cholesterol influx or efflux in our non-lipid loaded cell model system. Clearly, these results were obtained *in vitro*, and it is difficult to extrapolate them to

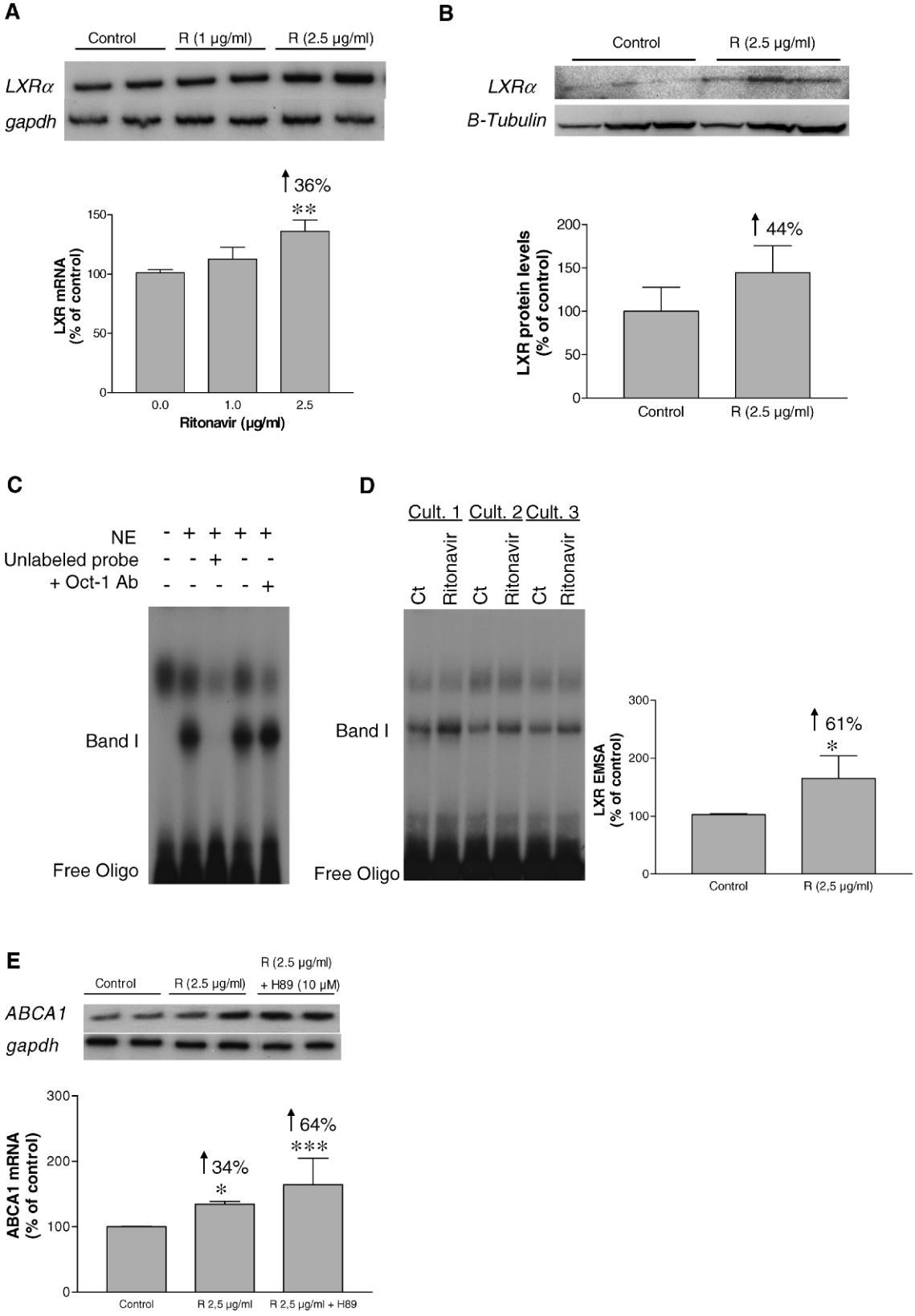

Figure 5. Ritonavir increases LXR mRNA, protein and binding activity, but not the cAMP-PKA pathway. **A**: Analysis of LXRα mRNA levels in THP-1 cells exposed to ritonavir 1 and 2.5 µg/ml during 24 h. **B**: Immunoblot analysis of LXRα in the nuclear fraction of THP-1 cells exposed or not to ritonavir 2.5 µg/ml during 24 h. Bands were quantified, normalized to the β-tubulin signal and the change in protein levels was calculated in comparison with the control situation (100%). **C**: EMSA assay showing the binding of THP-1 nuclear extracts (NE) to a LXRE oligonucleotide, forming a single specific band. **D:** Representative EMSA autoradiography showing the specific band formed with nuclear extracts from control

the *in vivo* situation in humans. Moreover, other PI-induced metabolic abnormalities (dyslipidemia, lipodystrophy and insulin resistance) may be considered as pro-atherogenic, but their contribution could be relatively minor compared to the effects of HIV infection itself (46).

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and ritonavir-treated THP-1 cells. On the right side of the figure, a bar plot shows the band intensity (in % of control) for control and ritonavir-treated cells. Each bar represents the mean ± SD of values from 3 independent cultures. **E:** Analysis of ABCA1 mRNA levels in THP-1 cells incubated with ritonavir 2.5 μg/ml alone or combined with the PKA inhibitor H89 (10 μM). RNA were analyzed by RT-PCR. Representative autoradiograms are shown. Bands were quantified, normalized to the gapdh signal and the change in mRNA levels was calculated in comparison with the control situation (100%). Data are expressed as mean ± SD of 3 independent experiments. * $P < 0.05$ and *** $P < 0.001$ versus corresponding control cells (ANOVA test).

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