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In Vitro Modulation of Peroxisome Proliferator-activated Receptor-γ and Its Genes by C-Reactive Protein. Role of Atorvastatin

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Background and Aims. C-reactive protein (CRP) serves not only as a biomarker for the risk of cardiovascular disease and underlying inflammation but also functions as an active mediator of atherosclerosis by promoting activation of endothelial cells and monocytes. Peroxisome proliferator activated receptor-gamma (PPAR-γ) transcription factor has been recognized to regulate the expression of many genes involved in inflammation, lipid metabolism and vascular remodeling. Therefore, in the present study we tried to explore the role of CRP as a possible mediator of atherosclerosis by determining its effect on PPAR-γ and its effector genes, i.e., liver X receptor-α (LXR-α) and matrix metalloproteinase-9 (MMP-9) in THP-1 cells.

Methods. Semi-quantitative RT-PCR was used to determine mRNA expression.

Results. CRP upregulates the expression of PPAR-γ and LXR-α at lower doses (5–25 μg/mL), which were further declined at higher doses (50–100 μg/mL). However, a dose-dependent increase was observed for MMP-9 expression. Atorvastatin (10–20 μM) was able to significantly accelerate the CRP-induced expression of PPAR-γ and LXR-α and attenuate MMP-9 expression.

Conclusions. For the first time we demonstrate that CRP modulates PPAR-γ and its effector genes and reinforces the mechanistic link of CRP as a possible mediator in atherosclerosis and also advocate atorvastatin as a therapeutic modality. © 2010 IMSS. Published by Elsevier Inc.

Key Words: C-reactive protein, PPAR-γ, LXR-α, MMP-9, Atorvastatin.

Introduction

Inflammation plays a central and pivotal role in atherosclerosis from its inception onwards (1). Nuclear receptors like peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs) have been implicated in the modulation of the expression of genes involved in inflammation and lipid metabolism in macrophages. Activation of macrophages in atheromas lead to release of vasoactive molecules such as nitric oxide (NO), endothelins (ETs), several eicosanoids, reactive oxygen species (ROS) (2) and secretion of proteolytic enzymes (e.g., matrix metalloproteinases (MMPs) that degrade extracellular matrix. The loss of matrix components subsequently leads to destabilization of the plaques and poses an increased risk for plaque rupture and thrombosis (3–4).

Considerable evidence has emerged to indicate that, in addition to inducing genes involved in reverse cholesterol transport, these nuclear receptors reciprocally repress a set of inflammatory genes after bacterial LPS, TNF-α or IL-1β stimulation (3,5). Inflammation also regulates the production of the acute phase proteins such as C-reactive protein (CRP), fibrinogen and serum amyloid A (1). CRP is not only a biomarker for atherosclerotic events (6) but a potent vasoactive mediator to promote atherogenesis by activating various inflammatory genes in macrophages including matrix metalloproteinases (MMPs), inflammatory chemokines and cytokines, adhesion molecules, tissue factor, etc. (7–11).

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3-Hydroxy-3-methylglutaryl coenzyme A (HMGCoA) reductase inhibitors (statins) are potent inhibitors of cholesterol biosynthesis. Recent *in vitro* and *in vivo* studies have shown statins have several pleiotropic effects that include decreasing the production of reactive oxygen species (ROS) (12), enhancing the levels of endothelial cell nitric oxide synthases (eNOS) (13), and inhibiting vascular smooth muscle cell (VSMCs) proliferation, as well as exhibiting antithrombotic and anti-inflammatory effects (14). Statins have been shown to stabilize atherosclerotic plaques and prevent coronary artery disease (CAD) events (15−16). It has also been demonstrated that statins increase PPARγ and LXRα mRNA expression and protein levels in human umbilical vein endothelial cells, hepatocytes and monocytes (17−18).

In this study we investigated how CRP (as a possible mediator in atherosclerosis/inflammation) modulates the expression of PPARγ and LXRα in human THP-1 monocytic cell line. Keeping in mind the fact that gene coding for MMP-9 is specifically downregulated by these nuclear receptors at the transcriptional level (19−20), we also determined the effects of CRP on the expression of MMP-9. Further, atorvastatin was explored as a potential therapeutic modality on the expression of these genes in the presence of exogenous CRP as an inflammatory stimulus.

Materials and Methods

Chemicals

All chemicals and reagents used in the experiments were of cell culture and molecular biology grade and were purchased from the following companies (Sigma, St Louis, MO, USA); Fermentas, Inc, St. Leon Rot Germany); Hi-Media, Mumbai, India), etc. The commercial recombinant *E. coli*-derived CRP preparation (236608, Calbiochem, La Jolla, CA) was used and sodium azide was removed as described previously (11). Bacterial endotoxin levels were determined using E-toxate kit (ET0100, Sigma). All reagents used in the experiments had endotoxin levels <0.125 IU/mL. E-toxa clean (E9029−500G, Sigma), along with autoclaving (121°C for 1 h) and dry heat (250°C for 3 h), was used to remove endotoxin contamination from glassware and water (11). Atorvastatin was purchased commercially from Cipla Pvt. Ltd. (Mumbai, India) (21).

Cell Culture

We used a monocytic cell line, the cells of which are often used as fair substitutes for simulating human monocytes/macrophages in analyzing the production of anti-inflammatory and inflammatory mediators/cytokines in response to various inflammatory stimuli. For the cell culture experiments, acute monocytic leukemia cell line, THP-1 was procured from NCCS (Pune, India). Cells were maintained in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% FCS in 5% CO_2 at 37°C (11,22).

Cell Viability and Cell Proliferation

Cell viability and cell proliferation of THP-1 cells was determined by trypan blue exclusion and cell proliferation assays (11647229001, Roche Diagnostics, Indianapolis, IN) respectively.

RNA Isolation and RT-PCR

Semiquantitative RT-PCR was performed to determine mRNA expression of PPAR-γ, LXR-α and MMP-9 in THP-1 cells. Total cellular RNA was isolated and was further reverse transcribed to cDNA using random hexamers (K1162, Fermantas). cDNA was then amplified by PCR using human PPAR-γ, LXR-α, MMP-9 and β-actin specific primer pairs (Table 1). The levels of mRNA expression of each gene were calculated as described previously (11,22) and expressed in terms of folds increase or decrease.

Effect of CRP on PPAR-γ, LXR-α and MMP-9 Gene Expression

THP-1 cells were incubated with different doses of recombinant CRP (5−100 µg/mL) for different time periods, i.e., 6, 24 and 48 h. During standardization of the dose of CRP and the time for maximum expression of the PPAR-γ, LXR-α and MMP-9 genes (time- and dose-dependent experiments) we found that there was no significant alternation in the expression of these genes at 3, 6, 9 or 12 h in the presence of different doses of CRP (data not shown). Thereafter, we have taken 6 h as a representative time period for further experiments.

Table 1. Characteristics of primers used for RT-PCR

Gene	Primer	Sequence	Fragment length (bp)	PCR cycles
PPARγ	Forward	5′-GCA GTG GGG ATG TCT CAT AAT GC-3′	297	32
	Reverse	5′-CAG GGG GGT GAT GTG TTT GAA-3′		
LXRα	Forward	5′-CAGAGAGGAAGCCAGGATGCC-3′	171	35
	Reverse	5′-GAGCGCCGGTTACACTGTTGC-3′		
MMP-9	Forward	5′-GCG GAG ATT GGG AAC CAG CTG TA-3′	208	22
	Reverse	5′-GAC GCG CCT GTG TAC ACC CAC A-3′		
β-actin	Forward	5′-GAATTGCTATGTGTCTGGGT-3′	257	22
	Reverse	5′-CATCTTCAAACCTCCATGATG-3′		

Effect of Atorvastatin on CRP-induced Expression of PPAR-γ, LXR-α and MMP-9

Time- and dose-dependent experiments were performed with different doses of atorvastatin (0–20 μM) in presence or absence of CRP for different time periods i.e., 6, 24 and 48 h. Cells were harvested and mRNA expression of PPAR-γ, LXR-α and MMP-9 genes was determined.

Statistical Analysis

Results are expressed as mean ± SD of at least three independent experiments. ANOVA was applied to test the level of significance for the expression among different time intervals and doses using post-hoc analysis with Bonferroni correction. A value of $p < 0.01$ was considered as highly significant and all values of $p < 0.05$ were considered as significant. All the analyses were performed using SPSS v.14.0.

Results

Cell Viability and Cell Proliferation

CRP (100 μg/mL) had no significant effect on the viability and proliferation of cells up to 48 h, whereas it was cytotoxic at 72 h. However, CRP at a dose of 150 μg/mL showed a significant decrease in cell viability and proliferation of cells at 48 h. A minimal dose of CRP, i.e., 25 μg/mL, was able to adversely affect cells at 72 h both in terms of viability and morphology. No morphological abnormalities were found in cell cultures up to 48 h with 100 μg/mL CRP (data not shown). Based on these observations, we used CRP at a maximum dose of 100 μg/mL and the experiments were carried out for only 48 h (Figure 1).

We observed that there was no change in the percentage of viability of THP-1 cells when treated with different doses of atorvastatin up to 40 μM until 48 h. A similar pattern was observed for cell proliferation with similar doses of atorvastatin until 48 h. At 72 h, THP-1 cells did not show any significant ($p > 0.05$) change in the percentage of viability of THP-1 cells up to 10 μM dose of atorvastatin, whereas higher doses of atorvastatin, i.e., 20 μM and 40 μM, caused a significant decrease in the percentage of viability and cell proliferation at 72 h ($p < 0.05$). No morphological abnormalities were found in cell cultures up to 48 h with a 40-μM dose of atorvastatin (data not shown). Thereafter, the experiments were carried out only up to 48 h (Figure 1).

Effect of CRP on PPAR-γ, LXR-α and MMP-9 Gene Expression

We observed that mRNA expression of both genes (PPAR-γ and LXR-α) was augmented 3-fold at a dose of 5 μg/mL of CRP at 24 and 48 h as compared to control THP-1 cells ($p < 0.01$). Expression of PPAR-γ and LXR-α was found to be maximal at a dose of 5 μg/mL CRP at 24 h compared to the expression at 6 h and 48 h. This observed increase in the expression of these genes declined significantly with increasing doses of CRP at 24 and 48 h, i.e., an inverse relationship was observed in the expression of both genes with an increase in the dose of CRP. Further, expression of both genes showed a parallel expression of mRNA transcripts in response to CRP by THP-1 cells. All experiments were repeated three times with similar results (Figure 2).

No significant change was observed in the expression of MMP-9 gene in the presence of 5 μg/mL of CRP at any of the time intervals studied. CRP at a dose of 25 μg/mL caused a 2- to 2.5-fold increase in MMP-9 mRNA expression both at 24 and 48 h time ($p < 0.05$). No significant difference in mRNA expression levels of MMP-9 was observed with 50 μg/mL dose of CRP when compared with 25 μg/mL dose both at 24 and 48 h ($p > 0.05$). However, an increment in the dose of CRP to 100 μg/mL caused a maximum increase in mRNA expression at 24 h (Figure 2).

Although we observed that a dose of 5 μg/mL of CRP was able to elicit maximum expression of PPAR-γ and LXR-α, no effect in MMP-9 expression was observed with this dose. However, a dose of 25 μg/mL of CRP was able to induce expression of all these genes (although not maximum). Thus, keeping in mind the pleiotropic effects of statins, in the additional experiments with atorvastatin we used CRP at a concentration of 25 μg/mL.

Effect of Atorvastatin on CRP-induced Expression of PPAR-γ, LXR-α and MMP-9

Effect of atorvastatin on CRP-induced expression of PPAR-γ, LXR-α and MMP-9 genes are depicted in Figure 3. We observed no significant differences in the mRNA expression of PPARγ, LXRα and MMP-9 genes in presence of any dose of the atorvastatin studied when compared to the gene expression observed in presence of CRP (either 5 μg/mL or 25 μg/mL) at 6 h.

A significant increment in the CRP-induced expression of PPARγ and LXRα was observed at 24 and 48 h, respectively, with a 10-μM dose of atorvastatin. Further, we observed a significant increment ($p < 0.05$) in the expression of the PPARγ and LXRα genes when the expression was compared between 24 h and 48 h. Interestingly, we observed a more or less parallel trend in the expression of both the genes studied in the present study (PPARγ and LXRα) in the presence of atorvastatin.

We observed that atorvastatin at a dose of 10 μM at 24 h was significantly able to attenuate the CRP-induced expression of MMP-9 gene. No significant alteration in the expression of CRP-induced MMP-9 gene was observed with any dose of atorvastatin until 6 h ($p > 0.05$).

Because atorvastatin is a lipophilic drug, it was dissolved in dimethyl sulfoxide (DMSO); thus, an equivalent

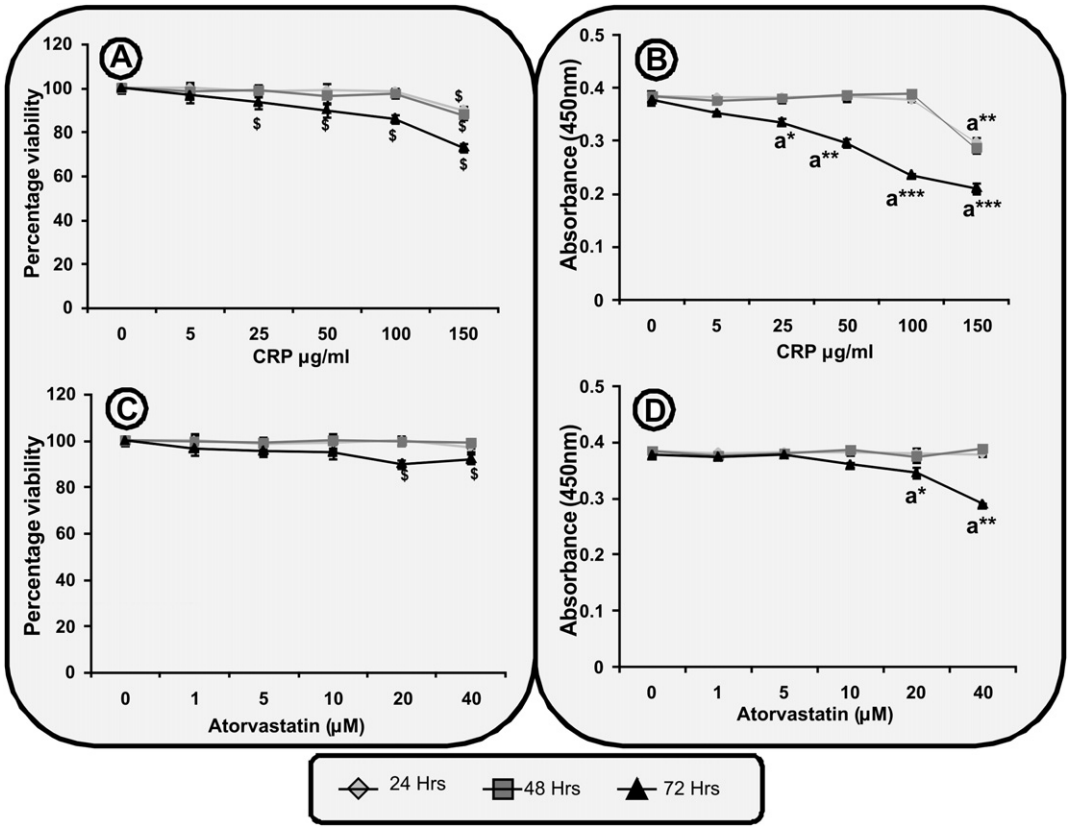

Figure 1. Data presented are Time- and dose-dependent effects of CRP (A,B) and atorvastatin (C,D) on cell viability (A,C) and cell proliferation (B,D). Data presented is mean ± SD of three experiments with similar results. Viability <95%; a = vs. basal; $^{*}p$ <0.05; $^{**}p$ <0.01; $^{***}p$ <0.001

amount of vehicle (0.05% DMSO) was also added to the control cells. No significant differences in the expression of these genes were observed either in the absence or presence of the vehicle (data not shown).

Discussion

Participation in both metabolic and inflammatory control is a common feature of a number of different nuclear receptor signaling pathways. PPARγ belongs to the nuclear receptor cum transcription factor family and has the unique distinction in that these genes not only control the expression of LXRα, CD36 and NFkB but also of the other effector genes that play a key role in the cooperativity that exists between oxidative and inflammatory processes like MMP-9 (23−24). It is in this context that results reported in the present study assume importance of existing inflammation and role of anti-inflammatory/inflammatory genes. In our study we observed that CRP (5−25 µg/mL) augmented the expression of PPARγ and its effector genes like LXRα and MMP-9. PPARγ and LXRα also have been documented to be the anti-inflammatory genes (23,25). The increased expression of PPARγ and LXRα could be viewed as a spontaneous compensatory or an adaptive response, which may exist to compensate or counteract the deleterious effects of inflammation and to counteract CRP-induced MMP expression to some extent. Further, increment in the doses of CRP up to 100 µg led to the decline in the expression of these two genes, whereas at the same time expression of inflammatory gene, i.e., MMP-9, was augmented (Figure 2).

A broad spectrum of inflammatory mediators like colony stimulating factors and oxidized low-density lipoprotein are shown to be highly expressed in the atherosclerotic lesion. These inflammatory mediators have been demonstrated to induce PPARγ expression *in vitro* (25). In the present study we demonstrated that another inflammatory intermediate, i.e., CRP, also induces the expression of PPARγ gene.

It has been reported in the literature that mild inflammation and viral infections can cause elevation of CRP levels at a range of 10−40 µg/mL (26), whereas active inflammation and bacterial infection may result in levels up to 40−200 µg/mL of CRP. In patients with chronic coronary artery disease, plasma CRP concentration increases mildly and the levels are <10 µg/mL in most cases (6). However, in the present study, CRP at a lower dose, i.e., 5 µg/mL, was able to elicit a significant effect on gene expression by THP-1 cells. At higher doses of CRP the expression of anti-inflammatory genes declined and the expression of inflammatory gene, i.e., MMP-9, increased. In an earlier

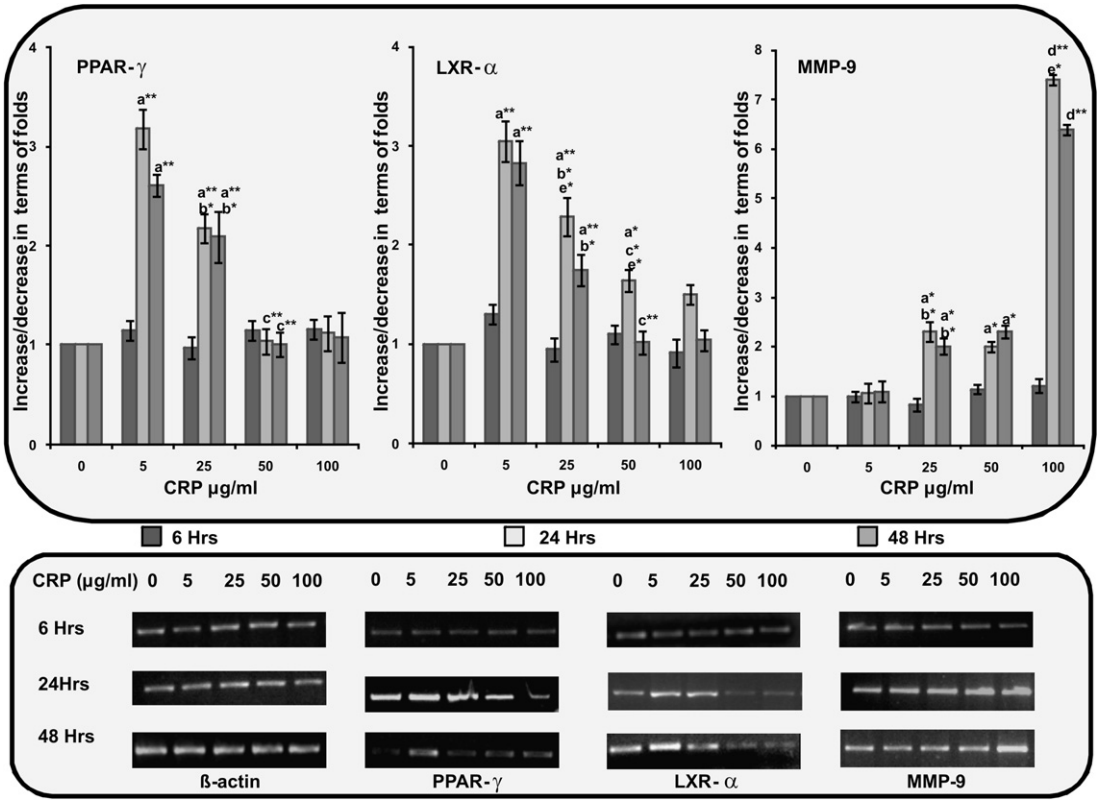

Figure 2. Time- and dose-dependent effects of CRP on mRNA expression of PPAR-γ, LXR-α and MMP-9 by THP-1 cells as determined by semiquantitative RT-PCR. Data presented are mean of three experiments with similar results. a = vs. basal; b = 5 vs. 25 µg/mL; c = 25 vs. 50 µg/mL; d = 50 vs. 100 µg/mL; e = 24 vs. 48 h; $^{*}p < 0.05$; $^{**}p < 0.01$.

report by Ricote et al. (27), increased expression of PPARγ in macrophage-derived foam cells of both early and intermediate human atherosclerotic lesions has been demonstrated. Inoue et al. (28) reported that LPS downregulated the expression of PPAR and upregulated the COX-2 expression in U937 cells and explained these findings by the role of a negative feedback loop mechanism involving PGD2. Although in the present study we did not elucidate the mechanism, keeping in mind that COX-2 is another effecter gene of PPARγ involved in inflammation like the MMP-9 gene, decreased expression of PPAR and increased MMP-9 expression at high doses of CRP could be explained, to some extent, by this mechanism. However, future studies are required in this direction. On the basis of our results, we hypothesize that CRP appears to be a mediator for macrophage activation, which leads to the modulation of PPARγ, LXRα and MMP-9 genes. Recently, it has been demonstrated that CRP induces the expression of MMPs and EMMPRIN genes *in vitro*, and thus plays an important role in plaque destabilization (8–10). However, in the present study, a maximum expression of MMP-9 gene was observed with a 100-µg/mL dose of CRP. Justification of such high doses of CRP (i.e., up to 100 µg) *in vivo* could be supported by a few other pathological studies reported in the literature. Torzewski et al. (29) demonstrated presence of CRP in human atherosclerotic lesions and a diffuse CRP staining in the fibromuscular layer of the intima and also demonstrated a positive CRP staining in the majority of macrophage foam cells. Burke et al. (30) showed a diffused CRP staining in the lipid core area and localized CRP staining in the cytoplasm of macrophages and also demonstrated a positive correlation between levels of CRP and the number of thin cap atheroma, suggesting the important role CRP may play to promote plaque vulnerability. All the evidences mentioned earlier clearly demonstrate that CRP possesses an inherent capacity to act as mediator in atherosclerosis and thus supports our hypothesis.

Further, our results demonstrated that statins upregulate the expression of genes coding for PPARγ and LXRα. Previous reports support our observations and demonstrate that statins have inherent property to act as anti-inflammatory agents (17,18,31). In the present study, atorvastatin at doses ranging from 10–20 µM was able to further augment the CRP-induced expression of PPARγ and LXRα genes and significantly attenuated MMP-9 expression. In a similar dose as used in the present study, atorvastatin was shown to inhibit the expression of resistin (17), lipoprotein lipase and endothelial lipase activity (32) in THP-1 cells. Apart from atorvastatin, in other *in vitro*

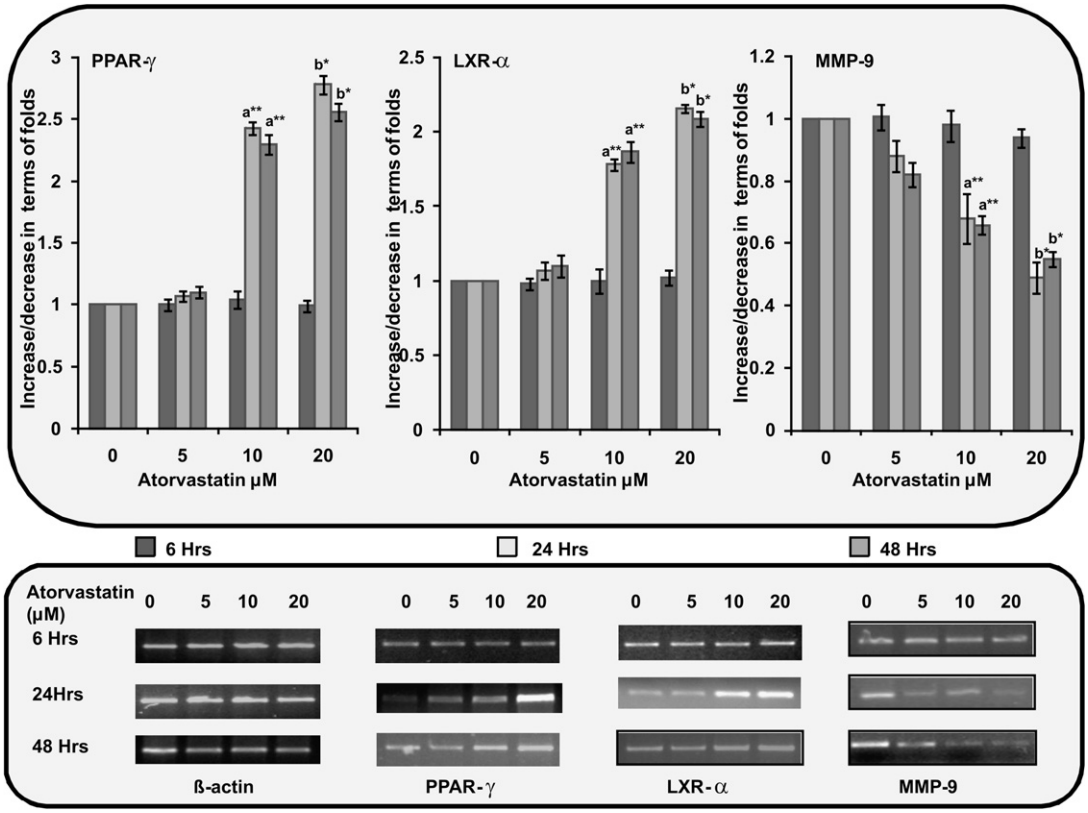

Figure 3. Time- and dose-dependent effects of atorvastatin on CRP-induced (25 μg/mL) mRNA expression of PPAR-γ, LXR-α and MMP-9 by THP-1 cells as determined by semiquantitative RT-PCR. Data presented are mean of three experiments with similar results. a = vs. basal; b = 10 vs. 20 μM; $^{*}p < 0.05$; $^{**}p < 0.01$.

studies, statins like simvastatin, pitavastatin, fluvastatin have also been reported to downregulate the CRP-induced expression of candidate genes involved in inflammation and atherosclerosis like IL-8 and resistin (33−34). Very recently, we have also reported that atorvastatin at similar doses (10−20 μM) was able to attenuate the CRP-induced expression of RAGE and its inflammatory ligand *in vitro* (11), and it was also able to attenuate lipopolysaccharide-induced expression of inflammatory genes in endometriotic stromal cells (21). Hence, our observations highlight and reconfirm the important anti-inflammatory properties of atorvastatin, which may be mediated by nonsteroidal products of the mevalonate pathway. Statins reduce not only cholesterol but also reduce intracellular pools of farnesyl and geranylgeranyl pyrophosphates, which are the metabolites of mevalonate. Many other proteins including small G proteins, e.g., Ras, Rho and Rac, are shown to be modified by isoprenoids. This modification is necessary for the proper localization and functioning of the proteins (34). Hiraoka et al. (35) reported the inhibition of ERK and RhoA by pitavastatin in THP-1 cells, and Masamura et al. (36) reported that mevalonate reversed the induction of thrombomodulin expression mediated by pitavastatin in endothelial cells. Although we did not study the effect of atorvastatin on signaling pathways, we can postulate that atorvastatin may inhibit the isoprenylation of small GTP-binding proteins such as Ras and Rho which, in turn, may affect their ability to interact with the plasma membrane.

The doses of atorvastatin used by our group in the present study in *in vitro* experiments have also been used by a number of other investigators worldwide to demonstrate different properties of atorvastatin *in vitro* (17,31). As far as the implications of the *in vitro* doses of atorvastatin are concerned, it is very difficult to compare the doses of the drug used in *in vitro* experiments to that used *in vivo*. Pharmacokinetic data on atorvastatin have shown that serum level of atorvastatin in human ranges between 0.002 and 0.2 μmol/l for a dose of 10−80 mg/day of atorvastatin (37−38). Hence, the concentrations of statins used in the present study and other *in vitro* studies may be slightly higher than these serum concentrations. Degraeve et al. (39) reported that higher concentrations are essential for the cholesterol independent effects of statins, i.e., inhibition of geranylgeranylation of proteins such as Rho GTPases. However, the concentration of the drug in blood depends on a variety of factors such as initial dose, absorption, varying metabolic rates and bioavailability. Another factor that must be considered when comparing *in vivo* and *in vitro* experiments is that these drugs are usually used

chronically *in vivo* as compared to their use in *in vitro* experiments where a biological effect needs to be assessed in a shorter time interval, thereby justifying the need for using higher doses *in vitro*.

Further, it is not clear what the concentration of the statin would be within the intima where macrophages reside, but one would expect it to be higher. These may be the reasons that frequently higher doses of drugs are used *in vitro* when compared to those found in the circulation. Despite the obvious limitations in the studies for using the drug *in vitro*, *in vitro* experiments also have an extra advantage of providing additional understanding and evidence regarding the phenomenon observed in a culture dish that has relevance to an *in vivo* environment. Thus, the favorable pleiotropic effects of statins may not only involve more than just a reduction in circulating CRP levels but may also negate the deleterious effects of CRP at the cellular level. Thus, statins may serve a dual purpose. First, they may provide a systemic anti-inflammatory effect, and second they may directly act at the tissue level inhibiting the CRP-mediated molecular pathways responsible for atherogenesis.

In the present study we used semiquantitative RT-PCR to determine the expression of genes instead of quantitative PCR and therefore is a limitation.

For the first time, findings of the present study provide sufficient evidence for potential links among CRP, nuclear receptors and MMP-9 in atherosclerosis and point out the possibility of treating inflammation-related diseases in a combinatorial approach that targets two or more of these receptors. In the future it would be worthwhile to explore the signaling cascades involved in the modulation of these genes and also the various genes that are controlled by these two transcription factors.

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Competing interests: The authors declare that they have no competing interests.

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