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Inhibition of C-reactive protein induced expression of matrix metalloproteinases by atorvastatin in THP-1 cells

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Abstract The role of CRP as a mediator in atherosclerosis and inflammation is being investigated worldwide. In the present study, the effect of CRP on matrix metalloproteinases (MMP)-1, 2, 9, and their tissue inhibitor (TIMP-1) gene expression in THP-1 monocytic cell line was investigated. Specific mitogen activated protein (MAP) kinase (ERK, p38, and JNK) inhibitors were used to elucidate the signaling pathways involved. Effect of atorvastatin was determined in the presence of CRP on the expression of genes. Time and dose-dependent experiments were performed in the presence of CRP. The results showed that the treatment of THP-1 cells with 100 μg of CRP/ml/10^6 cells for 24 h enhanced the expression of MMPs and TIMP-1 genes significantly. CRP upregulated the expression of these genes via FcγRII and utilized ERK signaling pathway to transduce signals. Atorvastatin was able to significantly attenuate CRP-induced MMPs expression and augmented TIMP-1 gene expression significantly. In conclusion, CRP is not only a risk marker for vascular events, but also directly involved in the mechanisms leading to remodeling and destabilization of atherosclerotic plaque. Also, atorvastatin serves as potential therapeutic modality to curb these harmful events.

Keywords C-reactive protein · MMPs · TIMP-1 · MAPK · Atorvastatin

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Introduction

Inflammation plays a key role in the pathogenesis of cardiovascular disease (CVD), acute atherothrombotic events, and atherosclerosis and also regulates the production of the acute phase proteins such as C-reactive protein (CRP), fibrinogen, and serum amyloid A [1, 2]. CRP is not only a biomarker for atherosclerotic events [3] but a potent vasoactive mediator to promote atherogenesis. In monocytes, CRP induces the production of inflammatory cytokines, matrix degrading enzymes, and promotes monocyte chemotaxis and tissue factor expression [4–8]. In endothelial cells, CRP increases the expression of cell adhesion molecules, chemokines, andendothelin-1 (ET-1), decreases endothelial nitric oxide synthase (eNOS) expression and activity, and augments monocyte-endothelial cell adhesion [9–11]. In VSMCs, CRP promotes its migration and proliferation and increases the production of reactive oxygen species (ROS) [12]. Atherosclerotic plaques are characterized by a lipid core covered by a fibrous cap composed of vascular smooth muscle cells (VSMCs) and extracellular matrix These plaques have chronic inflammatory infiltrates of macrophages and lymphocytes [2, 13, 14].

Many pathophysiological mechanisms contribute to the rupture of plaque such as inflammation, prothrombotic and thrombotic activity, shear stress, endothelial responsiveness to dilatation, collagen degradation, intraplaque angiogenesis, and the morphological characteristics of the plaque itself [14, 15]. The stability and the strength of the atherosclerotic fibrous cap is the result of a dynamic process between the production of collagen and its degradation by specific proteases [16]. Connective tissue matrix proteins are degraded by a range of proteases, the most widely studied of which are the metalloproteinase family (MMPs) [17, 18]. Although several cell lines in the plaque,

including smooth muscle cells and basophils, produce metalloproteinases, the major source is the activated macrophage [19]. TIMPs are proteins which regulate the connective tissue metabolism by forming a high affinity, irreversible complexes with the active form of the MMPs, rendering them inactive [20]. The presence of proinflammatory cytokines and other inflammatory mediators within the micro-environment of an atherosclerotic plaque leads to an imbalance between the MMPs and their tissue inhibitors (TIMPs), which renders the plaque prone to rupture [20]. Interstitial collagenase (MMP-1), gelatinase A (MMP-2), and gelatinase B (MMP-9) are the metalloproteinases which are more markedly elevated in patients with coronary syndromes both within the atherosclerotic plaque [21] and into the peripheral circulation [22, 23].

Mitogen-activated protein kinases (MAPKs; ERK, p38, and JNK) are likely to play crucial roles in the genetic response of many components of the cardiovascular system in the disease process. These kinases transduce signals in response to cellular stress such as inflammatory cytokines, ischemia, reversible ATP depletion, heat shock, endotoxin, and genotoxic stress. MAPKs are activated in monocytes-macrophages in a variety of inflammatory settings [24–26]. Notably, ERK, p38, and JNK activation in these cells results in the expression of many of the cytokines and inflammatory molecules observed in atherosclerotic lesions [27]. MAPK signalling has been shown to mediate pro-inflammatory actions of CRP in multiple cell types during vasculature inflammation [4–8].

Recent in vitro and in vivo studies have shown that statins have several pleiotropic effects that include decreasing the production of reactive oxygen species (ROS), enhancing the levels of endothelial cell nitric oxide synthases (eNOS), inhibiting vascular smooth muscle cell (VSMCs) proliferation, and exhibit anti-thrombotic and anti-inflammatory effects [28]. Stabilization of atherosclerotic plaques is a new therapeutic goal that poses the challenge of identifying critical mechanisms for intervention [14]. In vitro studies have shown statins to modulate the CRP-induced expression of various genes involved in inflammation and atherosclerosis [8, 29, 30].

Therefore, in the present study, we have investigated the effects of CRP in modulation of MMPs (MMP-1, 2, and 9) and TIMP-1 in THP-1 monocytic cell line. MAP kinases were exploited as possible signaling pathways involved in the modulation of these genes by CRP. Further, atorvastatin was used as a therapeutic modality to know whether, it influences CRP-modulated effects on MMP-TIMP expression in vitro.

Materials and methods

Cell culture

The human leukemic monocytic cell line (THP-1) was obtained from the National Center for Cell Sciences (NCCS), Pune, India and was maintained in RPMI-1640 [8, 31]. Cell viability and cell proliferation of THP-1 cells was determined by trypan blue exclusion and cell proliferation assays (11647229001, Roche Diagnostics), respectively [8].

The commercial recombinant *E coli*-derived CRP preparation used was supplied in 20 mmol/l Tris, 140 mmol/l NaCl, 2 mmol/l $CaCl_2$, pH 7.5, and 0.05% (wt/vol) sodium azide (236608, Calbiochem) preparation was used. Sodium azide and endotoxin was removed as described previously [8]. CRP was dialyzed twice against Tris/NaCl/$CaCl_2$ buffer at 4°C. 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.

RNA extraction and RT–PCR studies

Semi-quantitative RT–PCR was performed to determine mRNA expression of MMP-1, 2, 9, and TIMP-1 in THP-1 cells using human specific primers (Table 1). Total cellular

Table 1 Characteristics of primers

Genes	Primer sequence
β-actin	Forward : 5′-CAT GTA CGT TGC TAT CCA GGC-3′
	Reverse: 5′-CTC CTT AAT GTC ACG CAC GAT-3′
MMP-1	Forward: 5′-GAG CAA ACA CAT CTG AGG TAC AGG A-3′
	Reverse: 5′-TTG TCC CGA TGA TCT CCC CTG ACA-3′
MMP-2	Forward: 5′-AGA TCT TCT TCT TCA AGG ACC GGT T-3′
	Reverse: 5′-GGC TGG TCA GTG GCT TGG GGT A-3′
MMP-9	Forward: 5′-GCG GAG ATT GGG AAC CAG CTG TA-3′
	Reverse: 5′-GAC GCG CCT GTG TAC ACC CAC A-3′
TIMP-1	Forward: 5′-CAT CCT GTT GTT GCT GTG GCT GAT-3′
	Reverse: 5′-GTC ATC TGG ATC TCA TAA GCG TGG-3′

RNA was isolated and reverse transcribed to cDNA using random hexamers. The levels of mRNA expression of each gene were expressed as percentage value or in terms of folds increase or decrease [8, 31].

Effect of CRP on expression of MMP 1, 2, 9, and TIMP-1

THP-1 cells were incubated with different doses of CRP (0–100 μg/ml) for different time intervals, i.e., 6, 24, and 48 h. After incubation, cells were harvested and processed for determining mRNA expression of MMP 1, 2, 9, and TIMP-1 genes.

Involvement of receptors (Fcγ Rs)

As per evidence in the literature, FcγRs, i.e., I, II, and III are the major receptors for CRP in leukocytes. Various studies have demonstrated the Fcγ*R*II (CD32) and RI (CD64) as high and low affinity receptors of CRP. Expression of FcγRIII (CD16) is absent in THP-1 cells [5, 32]. In order to determine the role of these receptors in the regulation of MMPs (1, 2, and 9) and TIMP-1 by CRP in THP-1 cells, we performed blocking experiments. For this, THP-1 cells (10^6 cells/ml) were incubated with 50 and 100 μg/ml of *FcγRI* (*CD64*) (*Cat No AF1257*, *R&D Systems*, *USA*) and *FcγRIIa* (*CD32*) (*Cat No MAB1330*, *R&D Systems*, *USA*) monoclonal antibodies separately 2 h prior to the stimulation with CRP. Thereafter, mRNA expression of MMP-1, 2, 9, and TIMP-1 was determined at 24 h. Isotype antibody for the anti-CD32 (*Cat No MAB1460*, *R&D Systems*, *USA*) was used as control.

Involvement of ERK, p38 and SAPK/JNK signaling pathways

For elucidating the signaling pathways involved in the induction of these genes by CRP, we used specific inhibitors for the ERK (PD98059, Sigma), p38 (SB203580, Sigma), and SAPK/JNK (SP600125, Sigma) pathways in the presence or absence of CRP. For this, THP-1 cells (10^6 cells/ml) were cultured in the presence of different concentration of inhibitors (0–80 μM) 2 h prior to the stimulation with CRP.

Effect of atorvastatin on expression of MMPs (1, 2, and 9) and TIMP-1 in the presence or absence of CRP

Time and dose-dependent experiments were performed with different doses of atorvastatin (0–80 μM) in the presence or absence of CRP for different time periods (6, 24 and 48 h). Cells were harvested at different time intervals and mRNA expression of MMP-1, 2, 9, and TIMP-1 genes was determined.

Results

Effect of CRP on MMP 1, 2, 9, and TIMP-1 expression in THP-1 cells

The mRNA expression of these genes did not change significantly with different doses of CRP at 6 h ($P > 0.05$). However, at 24 and 48 h a dose-dependent increase was observed in the presence of CRP on MMP-1, 2, and 9 gene expressions.

CRP in a dose of 25 μg/ml caused a 2–2.5 folds increase in all the three MMPs mRNA expression (MMP-1, 2, & 9) both at 24 and 48 h time ($P < 0.05$). No significant difference in mRNA levels in MMP-9 was observed with 50 μg/ml CRP dose, when compared with 25 μg/ml CRP dose both at 24 and 48 h ($P > 0.05$), whereas, a gradual and significant increase was observed for MMP-1 and MMP-2 gene expression ($P < 0.05$). However, 100 μg/ml dose of CRP caused maximum mRNA expression at 24 h. No significant change was observed in the expression of these genes in the presence of 5 μg/ml of CRP at any of the time intervals studied (Fig. 1).

A biphasic effect of CRP was observed on TIMP-1 gene expression both at 24 and 48 h. No significant change was observed in the expression of TIMP-1 at a dose of 5 μg/ml of CRP at 24 and 48 h, whereas, an increment in CRP dose to 25 μg/ml caused approximately two folds increase in the mRNA expression of TIMP-1 by THP-1 cells at both 24 and 48 h ($P < 0.01$). The enhanced expression of TIMP-1 was reduced equivalent to the basal expression when THP-1 cells were incubated in the presence of a higher dose of CRP, i.e., 50 μg/ml both at 24 and 48 h. Further, increment in the CRP dose to 100 μg/ml caused a reversal of TIMP-1 gene expression as observed with 50 μg/ml dose and resulted in an enhanced expression of TIMP-1 mRNA up to threefold increase both at 24 and 48 h ($P < 0.01$). However, a comparison of the levels of mRNA expression of TIMP-1 showed no statistically significant difference between these two time intervals ($P > 0.05$; Fig. 1).

The results of these time- and dose-dependent experiments revealed that CRP in a dose 100 μg/ml had maximum effect on the expression of all the genes under consideration; therefore, in further experiments we used CRP in a maximum dose of 100 μg/ml.

FcγRII involved in CRP up-regulated expression of MMP 1, 2, 9, and TIMP-1

Results of the blocking experiments are shown in Table 2. The data obtained from these experiments clearly demonstrated that anti-CD32 antibody blocked CRP-stimulated expression of test genes at both doses, i.e., 50 and 100 μg/ml of antibodies and the effect was dose-dependent. However,

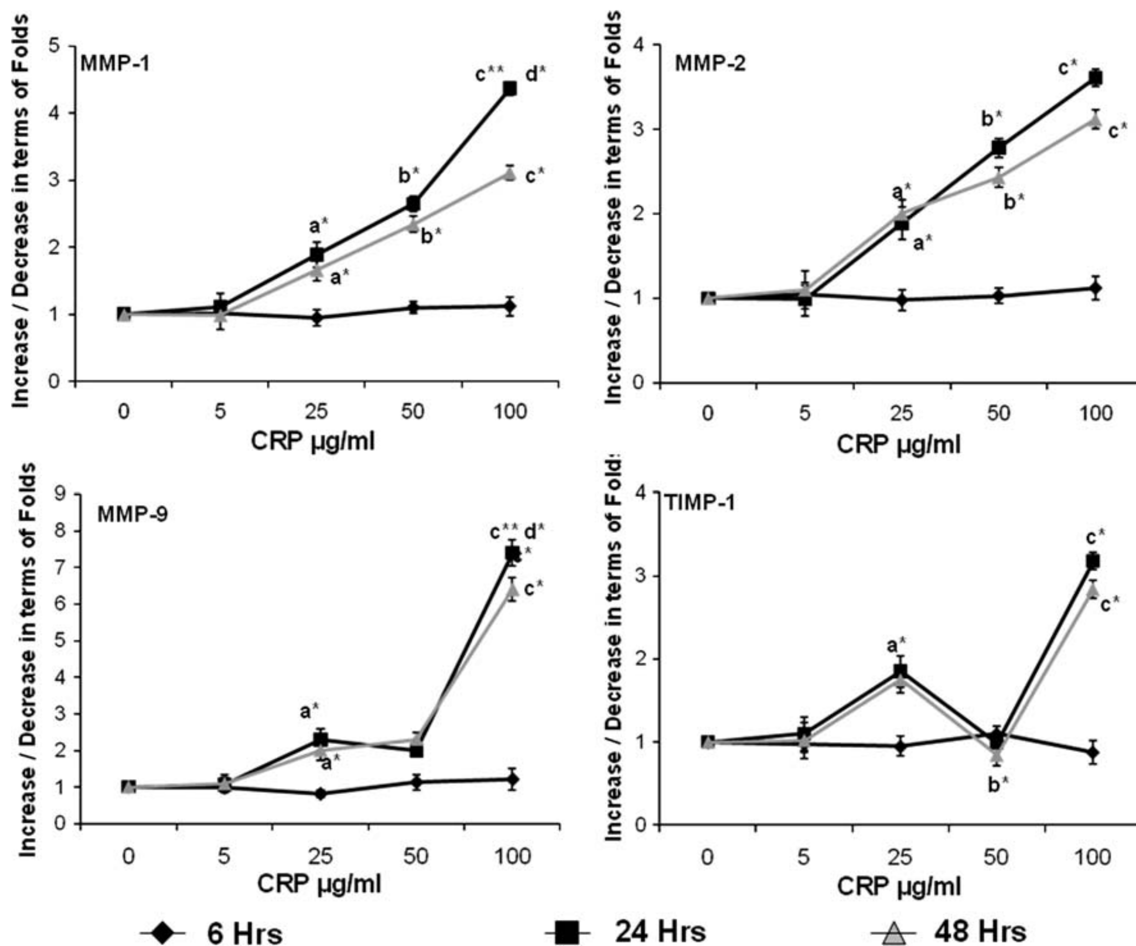

Fig. 1 Time and dose-dependent effects of C-reactive protein on expression of MMP-1, MMP-2, MMP-9, and TIMP-1 genes by THP-1 monocytic cell line by semi-quantitative RT–PCR. a = vs basal expression; b = 25 vs. 50 µg; c = 50 vs. 100 µg; d = 24 vs. 48 h; * $P < 0.05$; ** $P < 0.01$

Table 2 Percentage inhibition of CRP-induced gene expression by THP-1 cells in the presence of anti-CD64 and anti-CD32 antibody

Antibodies	MMP-1 (%)	MMP-2 (%)	MMP-9 (%)	TIMP-1 (%)
Anti CD64 (50 µg/ml)	13	18	14	16
Anti CD64 (100 µg/ml)	24	26	19	23
Anti CD32 (50 µg/ml)	44	39	40	39
Anti CD32 (100 µg/ml)	59	60	56	58
Isotype control for anti CD32 (50 µg/ml)	2	3	3	<0
Isotype control for anti CD32 (100 µg/ml)	<0	<0	1	1

THP-1 cells were incubated with 50 and 100 µg/ml of FcγRI (CD64), FcγRII (CD32), and CD32 isotype antibodies separately, 2 h prior to stimulation with the CRP (100 µg/ml) and mRNA expression of RAGE and EN-RAGE genes were determined at 24 h

anti-CD64 antibody was found to be less effective in blocking CRP-stimulated gene expression. Thus, our results demonstrate that FcγRII (CD32) receptor is the main receptor through which CRP induces expression of MMPs (1, 2, and 9) and TIMP-1 genes in THP-1 cells.

Involvement of signaling pathways in CRP-induced expression of MMP 1, 2, 9, and TIMP-1

Figure 2 shows the results of the experiments elucidating the involvement of signalling pathways for CRP induced expression of MMPs and TIMP-1. Gene expression of MMPs was inhibited in the presence of PD98059 (i.e., inhibitor of ERK signalling pathways). Similar to observations for different MMPs, i.e., 1, 2, & 9, TIMP-1 expression was also inhibited significantly in the presence of ERK inhibitor only but the inhibition was observed at a relatively higher dose, i.e., 40 µM ($P < 0.05$). However, the expression was found to be unaltered in the presence of the other two inhibitors, i.e., SB 253850 and SP 600125 which were specific for p38 and JNK pathways, respectively, even at maximum dose of 80 µM used in the present study (Fig. 2). These results suggested that the CRP-

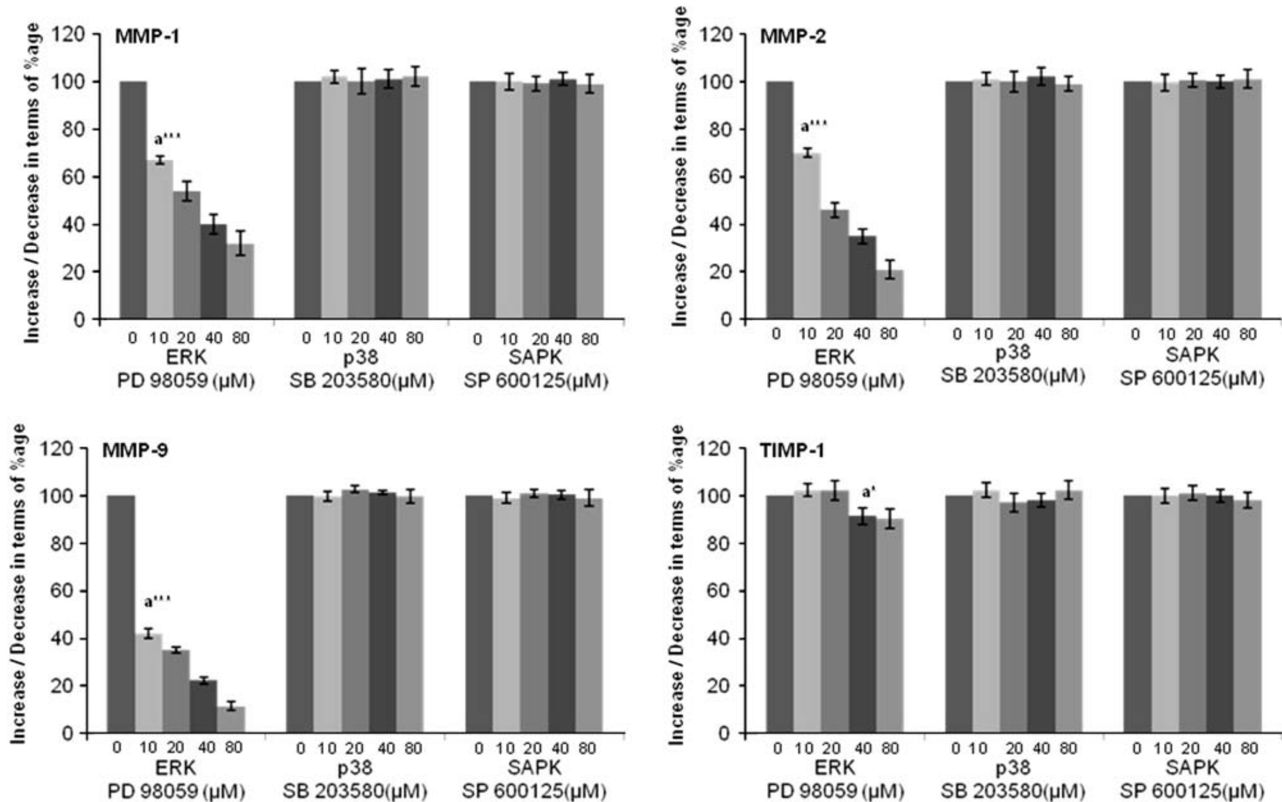

Fig. 2 Effect of different doses (0–80 µM) of inhibitors of MAPK pathways, i.e., PD 98059 [ERK (E)], SB 203580 [p38 (P)], SP 600125 [SAPK (S)] on CRP up-regulated gene expression of MMP-1, MMP-2, MMP-9, and TIMP-1 by THP-1 monocytic cell line as determined by semi-quantitative RT–PCR. a = vs basal; * $P < 0.05$; *** $P < 0.001$

induced expression of MMPs and their endogenous inhibitor TIMP-1 is dependent on ERK signalling pathway in THP-1 cells. Since, MAPK inhibitors were dissolved in dimethyl sulphoxide (DMSO), an equivalent amount of vehicle (0.05% DMSO) was also added to the control cells. However, we observed no significant differences in the expression of these genes either in the absence or presence of the vehicle (data not shown).

Effect of atorvastatin on CRP-induced gene expression of MMP 1, 2, 9, and TIMP-1

Treatment of THP-1 cells with atorvastatin attenuated the CRP-induced expression of MMPs in a time and dose-dependent manner, although, 5 µM dose of atorvastatin was unable to attenuate MMPs gene expression. No significant alteration in the expression of CRP-induced MMPs gene was observed with any dose of atorvastatin till 6 h ($P > 0.05$). Maximum decrease in the expression of MMPs gene was observed with 20 µM dose of atorvastatin at 48 h. Moreover, the difference in the attenuation of MMPs expression in the presence of 10 or 20 µM doses of atorvastatin at 24 and 48 h was statistically significant ($P < 0.05$) in case of MMP-1 and MMP-2 genes. However, we observed a more or less parallel trend in the attenuation pattern of CRP-induced MMPs gene expression by atorvastatin at these two time intervals (Fig. 3).

We observed, a significant increment in the CRP-induced expression of TIMP-1 in the presence of 20 µM of atorvastatin at 24 h ($P < 0.05$), however, there was no significant change at 48 h with the same dose ($P > 0.05$). Moreover, no significant alteration in the CRP-induced expression of TIMP-1 gene in the presence of other doses of atorvastatin at different time intervals ($P > 0.05$; Fig. 3) was observed. As atorvastatin is a lipophilic drug it was dissolved in DMSO as a vehicle and, therefore, an equivalent amount of vehicle was also added to the control samples (data not shown).

Discussion

The results of our in vitro studies revealed that CRP at a dose of 100 µg/ml was able to induce mRNA expression of MMPs (MMP-1, 2, & 9) up to 3–7 folds, thus, elucidating a potential mechanism by which CRP influences plaque vulnerability. Our data is also supported by an experimental study of Abe et al. [6] who reported that CRP

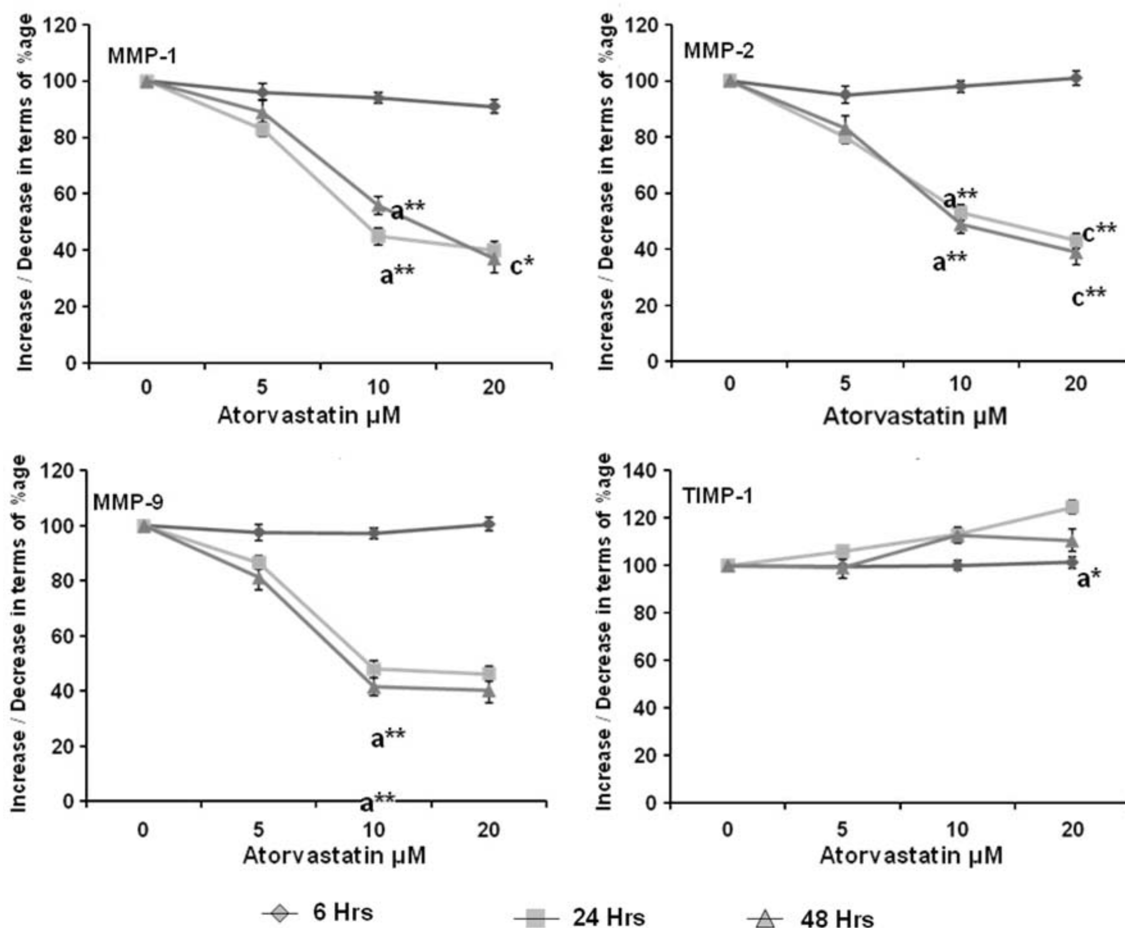

Fig. 3 Time and dose-dependent effect of atorvastatin (0–20 µM) on CRP up-regulated gene expression of MMP-1, MMP-2, MMP-9, and TIMP-1 by THP-1 monocytic cell line as determined by semi-quantitative RT–PCR. a = vs CRP up-regulated expression; $b = 5$ vs. 10 µM; $c = 10$ vs. 20 µM; $d = 24$ vs. 48 h; * $P < 0.05$; ** $P < 0.01$

induces MMP-9 gene expression and activity by increasing extracellular matrix metalloproteinase inducer (EMMPRIN) expression in rat macrophages. Studies in U937 histiocytes and in human aortic VSMCs have also demonstrated that CRP induces MMP-1 and MMP-2 gene expression by these cells, respectively [3, 33]. Recently, a study in healthy human volunteers [7] reported that CRP induces MMP-9 gene expression by peripheral blood mononuclear cells (PBMCs) obtained from these subjects and further that the observed induction in MMP-9 gene expression was through TNF-α and IL-1β production in vitro. All the evidences lined above clearly demonstrate that CRP possesses inherent capacity to induce the expression of MMPs and thus support our findings. Major et al. [34] reported that, whereas MMP-1 expression was coincident with VSMCs staining in the media and intima, MMP-9 expression coincided with CD68-positive macrophage staining. Together, all these findings support our view that CRP could be the main player in inducing the expression of these proteinases in human atherosclerotic plaques and thereby, accelerate rupture of weakened plaques. Collectively, our findings of augmented MMPs expression in vitro by CRP combined with the data obtained from the previous studies in the literature, profess the undisputable role of CRP and MMPs in pathophysiology of atherosclerosis.

As far as TIMP-1 levels are concerned, we observed a biphasic effect of CRP on TIMP-1 gene expression at both 24 and 48 h in THP-1 mononuclear cells and the effect was dose-dependent. Despite a biphasic effect, we observed maximum expression of TIMP-1 gene with 100 µg/ml dose of CRP at 24 h, which were similar to the effects of CRP observed on MMPs gene expression. Though, the reason for the biphasic expression of the TIMP-1 gene is not clear, the increased expression of TIMP-1 could be viewed as a spontaneous compensatory or adaptive response, which may exist to compensate or counteract the deleterious effects of CRP-induced MMP expression to some extent.

In rat peritoneal macrophages, Abe et al. [6] reported a time and dose-dependent increase in the TIMP-1 levels up

to 48 h which was maximum at 50 μg/ml CRP concentration. Rajagopalan et al. [35] documented an increased expression of MMPs and TIMPs in human atherosclerotic plaques and suggested that this increase could be due to the involvement of other biological mediators such as cytokines, ROS and cholesterol. In contrast, Williams et al. [5] and Doronzo et al. [33] observed no effect of CRP treatment on TIMP-1 expression in U937 histiocytes and human aortic VSMCs, respectively. The exact mechanism behind these variable observations on CRP-induced TIMP-1 expression remains to be elusive.

Although, CRP caused a parallel increase in TIMP-1 expression, concomitantly with MMPs, the adequate balance between MMPs and TIMP-1 may play an important role in the synthesis and degradation of extracellular matrix. Based on the increased ratio of MMPs to TIMP-1 in the present study, CRP appears to induce macrophage activation which may then weaken the extracellular matrix by proteolysis. On the basis of our results and scientific evidence existing in the literature, our data potentiates the evidence that, CRP is not simply associated with atherosclerotic events but is directly involved in the inflammatory pathogenesis of vascular alterations responsible for vascular remodeling and plaque destabilization.

In patients with chronic coronary artery disease, the plasma CRP concentration increases only mildly and the levels are <10 μg/ml in most of the cases [3]. However, in our in vitro studies, CRP at lower dose, i.e., 5 μg/ml was not able to elicit any significant effect on gene expression by THP-1 cells. Only higher doses of CRP, i.e., 25–100 μg/ml were able to upregulate expression of genes with atherosclerotic potential. The range of CRP which was used in the present study was higher than that observed in chronic inflammation. The higher dose of CRP as used in our study in vitro can be justified on the basis of observations of another study by Yasojima et al. [36], who reported that CRP mRNA levels in atherosclerotic plaques were 10.2- and 7.2-folds higher than those in normal artery and liver, respectively. They further demonstrated that CRP mRNA was expressed by SMCs and macrophages in the thickened intima of plaques. Therefore, it is very likely that higher local CRP levels in atherosclerotic lesions are needed to stimulate the expression of these genes by macrophages.

On the basis of our results, we hypothesize that CRP appears to be a mediator for macrophage activation and thereby, may act as a stimulus for plaque destabilization. Our data is further supported by few other pathological studies in the literature. Torzewski et al. [37] demonstrated the presence of CRP in the 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. [38] showed a diffuse 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.

The results of blocking experiments with antibodies to these receptors in our study, clearly demonstrate the active participation of FcγRII in the induction of MMP-1, 2, & 9 and their endogenous inhibitor by CRP in THP-1 cells. Our study is also in accordance with the earlier observations, where CRP has been shown to mediate its biological effects in endothelial cells and monocyte–macrophages via Fcγ*R*II and Fcγ*R*I [5, 7, 8].

The immunocytochemical detection of Fcγ*R*s in human atherosclerotic lesions highlights their importance [39]. Ryu et al. [40] demonstrated that FcγRII exclusively mediated CRP-induced intracellular ROS generation by human vascular smooth muscle cells. Recently, Devaraj et al. [41] documented that CRP-induces superoxide release in endothelial cells and promotes eNOS uncoupling and that these effects are reversed by inhibition of CD32 and CD64 receptor. In addition, in human monocytes, CRP has been shown to induce CCR2 expression via CD64 [32]. In a recent report, Wu et al. [42] reported that CRP-mediates tissue factor release via CD16 in vascular smooth muscle cells in vitro. Therefore, blocking or silencing of these receptors raises a possibility that these could to be exploited as therapeutic targets in inhibiting the deleterious effects of CRP on crucial genes involved in atherogenesis.

Our data clearly demonstrates that CRP up-regulates the expression of MMPs by active involvement of the ERK signalling pathways. No significant involvement of other signalling pathways (i.e. JNK and p38) was observed as far as CRP-induced expression of MMPs gene is concerned. Our results are substantiated by the findings of Williams et al. [5], who demonstrated that ERK1/2 signalling pathway is involved in CRP-induced expression of MMP-1 in U937 histiocytes. Role of ERK in up-regulation of MMPs (MMP-2 and MMP-9) has also been highlighted in another study by Doronzo et al. [33] in human VSMCs. Lin et al. [43] in their study reported that incubation of human vascular endothelial cells with CRP increases the gelatinolytic activity of MMP-2 and MMP-9 in a dose-dependent manner. These authors also demonstrated that treatment with anti-CD40 reversed the observed up-regulation in MMP activity, indicating that CRP exerts its direct pro-inflammatory effects via CD40-CD40L signaling pathway.

Our results also show that the CRP-induced expression of endogenous inhibitor of matrix metalloproteinases-1 (TIMP-1) is regulated via ERK signalling pathway alone, similar to the observations noticed for MMPs expression. To the best of our knowledge, our study is the first report which demonstrates that ERK signalling pathway is involved in

CRP-induced expression of TIMP-1 and that, the other two MAPKs (p38 and JNK) signalling pathways are not involved. Therefore, it becomes noteworthy here that MMP-TIMP axis is modulated by a common signalling pathway, i.e., ERK. Hence, inhibition of this signalling pathway could be of interest in designing the therapy to combat the adverse effects of CRP in lesion progression and plaque rupture. The observations obtained from the present study so far, pinpoint that MMP-TIMP axis co-operates with pro-inflammatory cytokines in an autocrine, paracrine, and endocrine manner at the site of atherosclerotic lesion and contributes to the pathophysiology of the disease.

Next, we tested whether atorvastatin by virtue of its anti-inflammatory potential could reverse the deleterious effects of CRP on these genes in THP-1 cells in a dose- and time-dependent manner. The results obtained so far demonstrate that, atorvastatin treatment significantly attenuated CRP-induced expression of MMP-1, 2 & 9 in THP-1 cells, whereas it enhanced the expression of TIMP-1 gene. 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.

As far as the literature is concerned, evidence related to the effects of atorvastatin on modulation of genes considered in the present study is lacking. In a similar dose as used in the present study, atorvastatin was shown to inhibit the expression of resistin [44], lipoprotein lipase, and endothelial lipase activity [45] in THP-1 cells. Apart from atorvastatin, in other in vitro studies, statins like simvastatin, pitavastatin, and fluvastatin have also been reported to downregulate the CRP-induced expression of candidate genes involved in inflammation and atherosclerosis like IL-8 and resistin [29, 30]. Our present and previous data regarding the effect of atorvastatin on inflammatory genes like MMPs, RAGE, EN-RAGE [8], and TIMP-1, further, highlights and reconfirms the important anti-inflammatory properties of atorvastatin which may be mediated by non-steroidal products of the mevalonate pathway.

In a recent study, Abe et al. [6] documented the inhibitory effects of fluvastatin on CRP-induced gene expression and activity of MMP-9 via inhibiting EMMPRIN (extracellular matrix metalloproteinase inducer). Nevertheless, Kibayashi et al. [30] showed that pitavastatin inhibited CRP-induced IL-8 production both at protein and mRNA levels in human aortic endothelial cells involving the inhibition of the three signalling pathways, i.e., ERK, p38, and JNK MAPKs. Statins like pravastatin, lovastatin, atorvastatin, and fluvastatin have been shown to inhibit the MAPK pathways in aortic endothelial cells (AoEC) [46], aortic SMCs [47], and cardiac myocytes [48]. As demonstrated previously in our study, CRP has been shown to mediate its effects through MAPKs signalling pathway. Though, we did not determined the direct effect of atorvastatin on MAPKs, but, from the previous studied mentioned above, one can speculate that atorvastatin exerted its beneficial effects on these genes by inhibiting MAPKs signalling pathway, thereby, negating CRP-induced effects.

On the basis of our results of the in vitro experiments, we infer that CRP itself participates in up-regulation of the genes with atherosclerotic potential and therefore, may have a profound role in maintenance of the inflammatory tone/status within the plaques and in destabilization of the atherosclerotic plaques. As MAPKs were observed to be the signaling pathways involved in modulation of these genes by CRP, therefore, targeted inhibition of these pathways could serve as therapeutic strategy. Atorvastatin as a therapeutic modality holds the promise to reduce the CRP-induced gene expression involved in inflammation and vascular remodeling.

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References

1. Paffen E, de Maat MPK (2006) C-reactive protein in atherosclerosis: a causal factor? Cardiovasc Res 71:30–39
2. Packard RS, Libby P (2008) Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. Clin Chem 54:24–38
3. Ridker PM, Rifai N, Rose L, Buring JE, Cook NR (2002) Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. N Engl J Med 347:1557–1565
4. Cermak J, Key NS, Bach RR, Balla J, Jacob HS, Vercellotti GM (1993) C-reactive protein induces human peripheral blood monocytes to synthesize tissue factor. Blood 82:513–520
5. Williams TN, Zhang CX, Game BA, He L, Huang Y (2004) C-reactive protein stimulates MMP-1 expression in U937 histiocytes through Fc[gamma]RII and extracellular signal-regulated kinase pathway: an implication of CRP involvement in plaque destabilization. Arterioscler Thromb Vasc Biol 24:61–66
6. Abe N, Osanai T, Fujiwara T, Kameda K, Matsunaga T, Okumura K (2006) C-reactive protein-induced up-regulation of extracellular matrix metalloproteinase inducer in macrophages: Inhibitory effect of fluvastatin. Life Sci 78:1021–1028
7. Nabata A, Kuroki M, Ueba H, Hashimoto S, Umemoto T, Wada H, Yasu T, Saito M, Momomura S, Kawakami M (2008) C-reactive protein induces endothelial cell apoptosis and matrix metalloproteinase-9 production in human mononuclear cells: implications for the destabilization of atherosclerotic plaque. Atherosclerosis 196:129–135
8. Mahajan N, Bahl A, Dhawan V (2009) C-reactive protein (CRP) up-regulates the expression of receptor for advanced glycation end products (RAGE) and its inflammatory ligand EN-RAGE in

THP-1 cells: inhibitory effects of Atorvastatin. Int J Cardiol. doi: 10.1016/j.ijcard.2009.01.008

9. Pasceri V, Wu HD, Willerson JT (2004) Modulation of vascular inflammation in vitro and in vivo by peroxisome proliferator-activated receptor-gamma activators. Circulation 101: 235–238
10. Venugopal SK, Devaraj S, Yuhanna I, Shaul P, Jialal I (2002) Demonstration that C-reactive protein decreases eNOS expression and bioactivity in human aortic endothelial cells. Circulation 106:1439–1441
11. Verma S, Li SH, Badiwala MV, Weisel RD, Fedak PWM, Li RK, Dhillon B, Mickle DAG (2002) Endothelin antagonism and interleukin-6 inhibition attenuate the proatherogenic effects of C-reactive protein. Circulation 105:1890–1896
12. Wang CH, Li SH, Weisel RD, Fedak PWM, Dumont AS, Szmitko P, Ren-Ke Li, Mickle DAG, Verma S (2003) C-reactive protein up-regulates angiotensin type 1 receptors in vascular smooth muscle. Circulation 107:1783–1790
13. Ross R (1999) Atherosclerosis—an inflammatory disease. NEJM 340:115–126
14. Libby P, Aikawa M (2002) Stabilization of atherosclerotic plaques: new mechanisms and clinical targets. Nat Med 8:1257–1262
15. Lee R (1997) The unstable atheroma. Arterioscler Thromb Vasc Biol 17:1859–1867
16. Davies MJ (1998) Reactive oxygen species, metalloproteinases, and plaque stability. Circulation 97:2382–2383
17. Galis ZS, Sukhova KG, Lark MAW, Libby P (1994) Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. J Clin Invest 94:2493–2503
18. Cleutjiens JPM, Kandala JC, Guarda E, Guntak RV, Weber KT (1995) Regulation of collagen degradation in the rat myocardium after infarction. J Mol Cell Cardiol 27:1281–1292
19. Newby AC (2006) Do metalloproteinases destabilize vulnerable atherosclerotic plaques? Curr Opin Lipidol 17:556–561
20. Raffetto JD, Khalil RA (2008) Matrix metalloproteinases and their inhibitors in vascular remodeling and vascular disease. Biochem Pharmacol 75:346–359
21. Loftus IM (2000) Increased matrix MMP-9 activity in unstable carotid plaques. A potential role in acute plaque disruption. Stroke 31:40–47
22. Kai H, Ikeda H, Yasukawa H, Kai M, Seki Y, Kuwahara F, Ueno T, Sugi K, Imaizumi T (1998) Peripheral blood of matrix metalloproteinases-2 and -9 are elevated in patients with acute coronary syndromes. J Am Coll Cardiol 32:368–372
23. Inokubo Y (2001) Plasma levels of matrix MMP-9 and TIMP-1 are increased in the coronary circulation in patients with acute coronary syndromes. Am Heart J 141:217
24. Force T, Pombo CM, Avruch AJ (1996) Stress activated protein kinases in cardiovascular disease. Circ Res 78:947–953
25. Kumar S, Boehm J, Lee JC (2003) p38 MAPKs: key signalling molecules as therapeutic targets for inflammatory diseases. Nat Rev Drug Discov 2:717–726
26. Muslin AJ (2008) MAPK signalling in cardiovascular health and disease: molecular mechanisms and therapeutic targets. Clin Sci 115: 203–218
27. Ballou SP, Lozanski G (1992) Induction of inflammatory cytokine release from cultured human monocytes by C-reactive protein. Cytokine 4:361–368
28. Blum A, Shamburek R (2008) The pleiotropic effects of statins on endothelial function, vascular inflammation, immunomodulation and thrombogenesis. Atherosclerosis. doi:10.1016/j.atherosclerosis.2008.08.022
29. Hu WL, Qian SB, Li JJ (2002) Decreased C-reactive protein-induced resistin production in human monocytes by simvastatin. Cytokine 40:201–206
30. Kibayashi E, Urakaze M, Kobashi C, Kishida M, Takata M, Sato A, Yamazaki K, Kobayashi M (2005) Inhibitory effect of pitavastatin (NK-104) on the C-reactive protein-induced interleukin-8 production in human aortic endothelial cells. Clin Sci 108:515–521
31. Mahajan N, Dhawan V, Sharma G, Jain S, Kaul D (2008) Induction of inflammatory gene expression by THP-1 macrophages cultured in normocholesterolemic hypertensive sera and modulatory effects of green tea polyphenols. J Hum Hypertens 22:141–143
32. Han KH, Hong KH, Park JH, Ko J, Kang DH, Choi KJ, Hong MK, Park SW, Park SJ (2004) CRP promotes MCP-1 mediated chemotaxis through upregulating CCR2 expression in human monocytes. Circulation 109:2566–2571
33. Doronzo G, Russo I, Mattiello L, Trovati M, Anfossi G (2005) C-reactive protein increases matrix metalloproteinase-2 expression and activity in cultured human vascular smooth muscle cells. J Lab Clin Med 146:287–298
34. Major TC, Liang L, Lu X, Rosebury W, Bocan TMA (2002) Extracellular matrix metalloproteinase inducer (EMMPRIN) is induced upon monocyte differentiation and is expressed in human atheroma. Arterioscler Thromb Vasc Biol 22:1200–1207
35. Rajagopalan S, Meng XP, Ramasamy S, Harrison DG, Galis ZS (1996) Reactive oxygen species produced by macrophage-derived foam cells regulate the activity of vascular matrix metalloproteinases in vitro. Implications for atherosclerotic plaque stability. J Clin Invest 98:2572–2579
36. Yasojima K, Schwab C, Mc Geer EG, Mc Geer PL (2001) Generation of C-reactive protein and complement components in atherosclerotic plaques. Am J Pathol 158:1039–1051
37. Torzewski J, Torzewski M, Bowyer DE, Fröhlich M, Koenig W, Waltenberger J, Fitzsimmons C, Hombach V (1998) C-reactive protein frequently co-localizes with the terminal complement complex in the intima of early atherosclerotic lesions of human coronary arteries. Arterioscler Thromb Vasc Biol 18:1386–1392
38. Burke AP, Kolodgie FD, Zieske A, Fowler DR, Weber DK, Jacob VP, Andrew F, Virmani R (2004) Morphologic findings of coronary atherosclerotic plaques in diabetics: a postmortem study. Arterioscler Thromb Vasc Biol 24:1266–1271
39. Ratcliffe NR, Kennedy SM, Morganelli PM (2001) Immunocytochemical detection of FcγR in human atherosclerotic lesions. Immunol Lett 2001(77):169–174
40. Ryu J, Lee CW, Shin JA, Park CS, Kim JJ, Park SJ, Han KH (2007) FcgammaRIIa mediates C-reactive protein-induced inflammatory responses of human vascular smooth muscle cells by activating NADPH oxidase 4. Cardiovasc Res 75:555–565
41. Devaraj S, Dasu MR, Singh U, Rao LVM, Jialal I (2008) C-reactive protein stimulates superoxide anion release and tissue factor activity in vivo. Atherosclerosis. doi:10.1016/j.atherosclerosis.2008.05.060
42. Wu J, Stevenson MJ, Brown JM, Grunz EA, Strawn TL, Fay WP (2008) C-reactive protein enhances tissue factor expression by vascular smooth muscle cells: mechanisms and in vivo significance. Arterioscler Thromb Vasc Biol 28:698–704
43. Lin R, Liu J, Gan W, Yang G (2004) C-reactive protein-induced expression of CD40–CD40L and the effect of lovastatin and fenofibrate on it in human vascular endothelial cells. Biol Pharm Bull 27:1537–1543
44. Ichida Y, Hasegawa G, Fukui M, Obayashi H, Ohta M, Fujinami A, Ohta K, Nakano K, Yoshikawa T, Nakamura N (2006) Effect of Atorvastatin on in vitro expression of resistin in adipocytes and monocytes/macrophages and effect of atorvastatin treatment on serum resistin levels in patients with type 2 diabetes. Pharmacol 76:34–39
45. Qiu G, Hill JS (2007) Atorvastatin decreases lipoprotein lipase and endothelial lipase expression in human THP-1 macrophages. J Lipid Res 48:2112–2122

46. Takata M, Urakaze M, Temaru R, Yamazaki K, Nakamura N, Nobata Y, Kishida M, Sato A, Kobayashi M (2001) Pravastatin suppresses the interleukin-8 production induced by thrombin in human aortic endothelial cells cultured with high glucose by inhibiting the p44/42 mitogen activated protein kinase. Br J Pharmacol 134:753–762
47. Negre AP, Van Viiet AK, Van Erck M, Van Thiel GC, Van Leeuwen RE, Cohen LH (1997) Inhibition of proliferation of human smooth muscle cells by various HMG-CoA reductase inhibitors; comparison with other human cell types. Biochim Biophys Acta 1345:259–268
48. Kashiwagi Y, Haneda T, Osaki J, Miyata S, Kikuchi K (1998) Mechanical stretch activates a pathway linked to mevalonate metabolism in cultured neonatal rat heart cells. Hypertens Res 21: 109–119