Atorvastatin Inhibits Functional Expression of Proatherogenic TLR2 in Arterial Endothelial Cells

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Key Words

Toll-like receptors • Atherosclerosis • Statins • Inflammation

Abstract

Background: There is growing evidence that TLR2 plays a role in the pathogenesis of atherosclerosis. It is highly expressed in endothelial cells in areas of disturbed blood flow, like plaques or vessel bifurcations, but laminar blood flow suppresses endothelial TLR2 expression and is therefore thought to be atheroprotective. We sought for means to also protect lesion prone sites from TLR2 over-expression and subsequent endothelial activation. **Methods:** Human coronary artery endothelial cells (HCAEC) were treated with atorvastatin (ATV) and TLR2 surface expression was determined by FACS analyses. Western blot analyses were used to explore the phosphorylation status of SP1. **Results:** ATV profoundly inhibited basal and stimulated endothelial TLR2 expression in a time- and dose-dependent manner. It also inhibited HCAEC activation by MALP-2. TLR2 surface expression was inversely correlated to SP1 serine phosphorylation and was casein kinase 2 dependent. **Conclusion:** We demonstrate that ATV can control over-expression of proinflammatory endothe-

lial TLR2 protein and TLR2-mediated endothelial activation. The mechanism involves casein kinase 2 and SP1 phosphorylation. ATV effects on endothelial cell TLR2 are comparable to those of laminar blood flow and might therefore also be atheroprotective.

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Introduction

Atherosclerosis represents the major cause of morbidity and mortality in Western society [1]. Epidemiologic evidence has demonstrated a relationship between microbial infection and disease, with experimental studies suggesting a causal role [2]. The inflammatory nature of this disease process is widely accepted; however, the precise components of the atherogenic pro-inflammatory cascade remain controversial [3]. TLRs as principal sensors of the innate immune system provide a mechanistic link between infection, inflammation, and atherosclerosis [4]. Additionally, the possibility of endogenous ligand activation of TLRs might provide sterile inflammation links to atherosclerosis susceptibility [5].

To date, 10 TLRs have been described in humans with TLR1-9 conserved between the human and the mouse. These pattern recognition receptors have been

identified within human atherosclerotic vessels and lesions [6]. Immunohistochemical analyses by Edfeldt et al. revealed TLR1, TLR2, and TLR4, especially in the endothelium and areas positive for macrophages [7]. TLR2 and TLR4 seemed therefore potentially important in the pathogenesis of atherosclerosis. Experimental studies identified a role for TLR4 as well as the common TLR downstream signaling adaptor MyD88 in atherosclerosis [8-10]. However, this role of TLR4 remains controversial. There is significant evidence that TLR2 has a predominant role in human atherosclerosis - even more so than TLR4 [11]. But some authors still question whether TLR2 could play a role in atherosclerosis [12]. However, Mullick et al. reported that complete TLR2 deficiency results in reduced atherosclerosis. Bone marrow (BM) transplantation experiments further revealed that loss of TLR2 expression from BM-derived cells did not influence disease progression, when no additional inflammatory stimuli were administered to the mice [13]. Previously, we reported in an *in vitro* study using human coronary artery endothelial cells (HCAEC), that enhanced endothelial TLR2 expression and activation occurs at areas of disturbed blood flow, such as the areas of lesion predilection within the aortic tree and coronary arteries. Laminar flow profoundly suppressed TLR2 expression in these endothelial cells and involved SP1 phosphorylation and casein kinase 2. It might be that laminar flow induces CK2 phosphorylation of SP1, which is then inhibited to bind to the TLR2 promoter region [14]. Recently, our *in vitro* study was confirmed *in vivo*. In LDLr(-/-) mice TLR2 expression was restricted to endothelial cells in regions of disturbed blood flow. At these sites leukocyte accumulation, lipid accumulation, foam cell generation and endothelial cell injury were all increased, and the expression of endothelial TLR2 contributed to early atherosclerotic processes in these lesion-prone areas [15].

Since we cannot change the architecture of the arterial vessels in humans, we sought for alternatives to suppress TLR2 expression at atherosclerotic lesion prone sites.

Materials and Methods

Cell culture

Human coronary artery endothelial cells (HCAEC; Clonetics®) were purchased from Lonza (Verviers, Belgium) and grown to confluence in full growth medium (EGM-2-MV) from the same company. Thereafter, the cells were transferred to 6-well plates for experiments. There was no difference in

TLR2 surface expression or responsiveness to various stimuli between cells of different passages (4-8). The medium used in experiments (EM) was EGM-2-MV, but without hydrocortisone. Cytotoxic effects of atorvastatin (ATV; gift from Pfizer Corporation, Vienna, AT) or its solvent DMSO at concentrations used in the experiments were excluded by the MTT test [16].

Cell stimulation experiments

With the exception of the time-course experiments, all cell stimulation experiments started after a 24h incubation period with ATV. Before stimulation with TNF α (Roche, Mannheim, Germany), LPS (*E. coli* O55:B5; List Biological Laboratories, Campbell, CA), or Macrophage-activating lipopeptide 2 (MALP-2) (Alexis Biochemicals, San Diego, CA), medium was changed to fresh EM. After stimulation, cells were then either stained for FACS analyses using PE-labeled anti-human E-selectin (PharMingen, San Diego, CA) or anti-human TLR2 monoclonal antibody 2932 (gift Peter S. Tobias, TSRI, LaJolla, CA), or cells were subjected to nuclear extraction, or whole cell lysate preparation. Cell activation was determined by measuring E-selectin surface expression in FACS analyses (FACS Calibur, BD, Franklin Lake, NJ). This adhesion molecule is barely expressed on resting HCAEC but rapidly and highly induced upon stimulation. The secondary antibody to anti-human TLR2 was a PE-labeled donkey anti-mouse IgG (Sigma-Aldrich, Vienna, Austria). In some experiments the selective protein kinase CK2 (formerly casein kinase II) inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB; EMD Biosciences, San Diego, CA) was used to inhibit SP1 serine phosphorylation. Cells were co-incubated with TBB and ATV for 24h and thereafter endothelial TLR2 expression was stimulated with TNF α before surface expression was again determined by FACS analyses.

Western blot analyses

Whole cell lysates (RIPA buffer) or nuclear extracts [17] (10 μ g per slot; measured with Pierce BCA protein assay kit) from HCAEC were electrophoresed in NuPAGE 4-12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (BioRad, Hercules, CA). After blocking in Tris-buffered saline with 5% non-fat dry milk and 0.1% Tween 20, the membranes were incubated with anti-human SP1 (Upstate Biotechnology Inc., Lake Placid, NY) for 2h at room temperature, followed by washing and incubation with secondary horseradish-peroxidase-linked goat anti-rabbit IgG (Sigma-Aldrich, Vienna, Austria). In some experiments antibodies were stripped with 1% SDS from the membranes, which were then reprobed with a rabbit polyclonal anti-phosphoserine (α PS) antibody (Chemicon, Temecula, CA). Thereafter, a densitometry analysis of the bands of interest was performed with ImageJ® software.

Statistical analyses

Data are expressed as mean and standard error of the mean of five independent experiments (individual cell preparations). Means were compared by Mann Whitney U Test after Kruskal-Wallis analysis of variance. Statistical analyses were performed using the StatView software package (Abacus Concepts, Berkeley, CA).

Fig. 1. (left) HCAEC were incubated for 24h with ATV (10 μ M) and then stimulated with TNF α (50 ng/mL) or LPS (100 ng/mL) for further 12h. TLR2 surface expression was determined by FACS analyses as percentage of TLR2 positive cells. Data are given as mean $\pm$ standard error of the mean. n=5. * $P < 0.05$; ** $P < 0.01$ versus respective control without ATV (Mann Whitney U test). (right) Histogram chart representing the FACS analysis of a single experiment. Cells were treated with ATV and TNF as described above and then analysed by FACS (5000 events were counted/sample). Data are given as the mean channel fluorescence (MCF).

Results

ATV inhibits TNF α - or LPS-induced HCAEC TLR2 surface expression

HCAEC were incubated in EM for 24h with ATV (10 μ M) and after washing, TNF α (50 ng/mL) or LPS (100 ng/mL) were added in fresh EM for further 12h. Thereafter, TLR2 surface expression was determined by FACS analyses. Preincubation of cells with ATV not only reduced basal TLR2 surface expression, but also inhibited TNF α or LPS inducible TLR2 expression (Fig. 1). Long term incubation of HCAEC with ATV at concentrations up to 100 μ M did not show any cytotoxic or growth inhibiting effect (MTT test, data not shown).

Time dependency and dose-response of the ATV effect

Pretreatment of HCAEC with various concentrations of ATV (0.01 - 50 μ M) for 24h showed a significant inhibition of TNF α -induced (12h) TLR2 expression at concentrations of 0.1 μ M and above. At 10 μ M of ATV the effect was complete and further increasing ATV did not show any other effect on TLR2 surface expression (Fig. 2A). Time-course experiments using ATV at 0.1 μ M revealed longer preincubation periods before TNF α stimulation could enhance the ATV effect. The maximum inhibition was seen after 24h; at this time point and beyond even 0.1 μ M of ATV completely inhibited TNF α -induced HCAEC TLR2 surface expression (Fig. 2B).

ATV reduces susceptibility of HCAEC to activation by MALP-2

After pretreatment for 24h with ATV (10 μ M), HCAEC were either stimulated with TNF α (10 ng/mL), LPS (100 ng/mL), or the selective TLR2 agonist MALP-2 (10 ng/mL and 100 ng/mL) for 6h. Thereafter, cell activation was determined by FACS analyses of surface E-selectin expression. ATV did not affect TNF α - or LPS-

Fig. 2. HCAEC were incubated with various concentrations of ATV (0.01 - 50 μ M) for 24h and then stimulated with TNF α (50 ng/mL) for 12h (A) or cells were pretreated with ATV (0.1 μ M) for an increasing time period (20min - 48h) and then they were stimulated with TNF α (50 ng/mL) for 12h (B). TLR2 surface expression was determined by FACS analyses as percentage of TLR2 positive cells. Data are given as mean $\pm$ standard error of the mean. n=5. * $P < 0.05$ versus respective control without ATV (Mann Whitney U test).

induced E-selectin upregulation, but inhibited cell activation by MALP-2 at both concentrations tested (Fig. 3A). ATV alone had no influence on cell surface E-selectin expression and incubation of HCAEC with ATV (10 μ M) up to 6h did not affect activation of the cells by MALP-2. As expected, MALP-2 stimulated E-selectin expression was suppressed after 12h of ATV treatment (Fig. 3B).

Fig. 3. HCAEC were pretreated with AVT (10 μ M) for 24h and then cells were stimulated with TNF α (10 ng/mL), LPS (100 ng/mL), or MALP-2 (10 ng/mL and 100 ng/mL) for 6h (A), or HCAEC were stimulated with MALP-2 (10 ng/mL) after pretreatment with ATV (10 μ M) for various time intervals (B). Cell activation was determined by measuring cell surface E-selectin expression with FACS as percentage of E-selectin positive cells. Data are given as mean $\pm$ standard error of the mean. $n=5$. n.s. not significant; * $P < 0.05$ versus respective control without ATV (Mann Whitney U test).

ATV induces SP1 phosphorylation in HCAEC

HCAEC were exposed to ATV (10 μ M) for 24h and after washing, cells were stimulated with TNF α (50 ng/mL) for 12h, lysed and nuclear extracts were prepared. Although ATV and TNF slightly decreased SP1 protein in nuclear extracts, no major changes in the abundance of the protein during the time of incubation could be observed (Fig. 4 left). When the blotting membranes were stripped and reprobed with an anti-phosphoserine antibody, it was observed that ATV induced serine phosphorylation of SP1. TNF α reduced SP1 serine phosphorylation, which was antagonized by ATV pre-treatment (Fig. 4 right).

Co-incubation of HCAEC with ATV and TBB restores TLR2 surface expression

HCAEC were co-incubated with ATV (10 μ M) and TBB (10 μ M) for 24h before cells were stimulated for TLR2 expression with TNF α (50 ng/mL) for 12h. As

Fig. 4. HCAEC were exposed to ATV (10 μ M) for 24h and then cells were stimulated with TNF α (50 ng/mL) for 12h. Thereafter, cells were lysed and nuclear extracts were prepared. Extracts were transferred to western blot analyses and membranes were probed with an anti-SP1 antibody (left panel) and after stripping of the same blotting membrane, this was reprobed with an anti-phosphoserine antibody (right panel). Bands were analysed with densitometry software (bottom). Shown is one representative blot out of three experiments.

Fig. 5. HCAEC were co-incubated with ATV (10 μ M) and TBB (10 μ M) for 24h and then cells were stimulated with TNF α (50 ng/mL) for 12h. TLR2 surface expression was determined by FACS analyses as percentage of TLR2 positive cells. Data are given as mean $\pm$ standard error of the mean. $n=5$. * $P < 0.05$ versus respective control without TBB (Mann Whitney U test).

expected ATV alone reduced the basal expression of TLR2 and TNF α enhanced the expression. Addition of TBB to HCAEC had no effect on basal expression of surface TLR2 and did not influence the TNF α -induced upregulation of the receptor. But TBB was able to restore the basal TLR2 expression and in part the TNF α triggered TLR2 expression that were both inhibited by ATV (Fig. 5).

Discussion

In addition to lipid lowering effects, HMG-CoA reductase inhibitors (statins) appear to have pleiotropic immunomodulatory and anti-inflammatory properties. Multiple experimental and clinical studies support this additional activity of these drugs. For example, statins attenuate low-dose LPS-induced inflammatory activation of blood vessels [18, 19]. Therefore, some of their pleiotropic effects might be mediated through innate immunity. In fact, this has been proven in human monocytes, where simvastatin and atorvastatin inhibited LPS-induced TLR4 and TLR2 upregulation [20, 21]. More recently, it was shown, that atorvastatin inhibits this TLR4 upregulation also in endothelial cell. This was mediated by direct and indirect inhibition of NF- κ B via inhibition of ERK phosphorylation [22]. However, the involvement of TLR4 in the pathogenesis of atherosclerosis is not well established [10], but TLR2 seems to be a major player in the initiation and progression of this inflammatory vessel wall disease. Mullick and coworkers clearly demonstrated that in hypercholesteremic mice complete TLR2 deficiency is atheroprotective, but TLR2 deficiency solely in bone marrow derived cells, like monocytes, does not influence disease [13]. Thus, suppression of TLR2 and TLR4 in monocytes seems not to be the atheroprotective mechanism of statins in hypercholesterolemia.

In vitro and *in vivo* studies confirmed that endothelial TLR2 is over expressed in areas of disturbed blood flow. This was also shown to be proatherogenic. On the other hand, laminar blood flow profoundly suppresses expression of TLR2 on endothelial cells [14, 15].

We investigated HCAEC under static conditions for TLR2 expression. A previous investigation revealed that for HCAEC disturbed flow and static conditions are mimetic of each other insofar as MALP-2 reactivity is concerned [14]. *In vivo*, plasma levels of 1 μ M of ATV can be achieved by treatment with 20 mg daily of ATV. Pretreatment of cells with ATV (0.1 μ M) led to a significant reduction in endothelial surface TLR2. This effect was augmented with increasing incubation time, but it was not caused by cytotoxicity (MTT test) or energy depletion of the cells, since surface E-selectin expression upon TNF α or LPS stimulation remained unaffected in the cells. E-selectin could therefore be used to monitor MALP-2 reactivity. Results showed a good correlation between surface TLR2 expression and reactivity of the cells to a specific TLR2 ligand agonist. But reduced reactivity was not due to direct inhibition of TLR2, since short term incubation of HCAEC with ATV did not affect MALP-2

activation. After 12h, when TLR2 goes down, MALP-2 lost most of its ability to stimulate the cells. Beyond that time point, further ATV treatment suppressed surface TLR2 levels to basal expression (which is usually very low in endothelial cells) and made cells almost unresponsive to MALP-2 stimulation. In endothelial cells ATV can diminish LPS- induced TLR4 upregulation by inhibiting NF- κ B activation, both directly and indirectly [22]. In our experiments ATV did not affect TNF- or LPS-induced E-selectin expression in HCAEC, and both, TNF or LPS, are known to induce NF- κ B activation. Of course we cannot exclude that ATV is affecting any marker of this pathway, but as it concerns E-selectin expression, this is unaffected by ATV.

The biological relevance of our finding is underscored by the fact that MALP-2 is one of the most potent TLR2 agonists [23]. SP1 elements regulate TLR2 promoter activity in the human myeloid THP-1 cell line [24]. Important for our study is SP1 phosphorylation by protein kinase CK2, which mainly phosphorylates serine in the zinc finger domain [25]. This inhibits SP1 DNA binding activity. In fact, in HCAEC serine phosphorylation of SP1 was inversely correlated to the DNA binding activity of SP1 [14]. Western blot analyses of HCAEC treated with ATV, TNF α , or both showed no major differences in the amount of intranuclear SP1 protein, but revealed that TNF α reduced SP1 serine phosphorylation and ATV restored it or even enhanced it. The status of SP1 phosphorylation inversely correlated to TLR2 cell surface expression. To prove, whether CK2 is involved, we explored the effects of the specific CK2-inhibitor TBB. CK2 blockade abolished effects of ATV on HCAEC TLR2 surface expression, confirming an involvement of CK2 not only in flow mediated effects on receptor expression, but also in ATV-induced SP1 serine phosphorylation with subsequent inhibition of TLR2 expression. The facts that ATV only partially restores SP1 phosphorylation reduced by TNF α and that protein kinase CK2 inhibition only partially restores ATV-induced suppression of TNF α -induced TLR2 expression are indicative for the involvement of additional downstream effectors.

Previous studies pointed out a key role of endothelial TLR2 in the pathogenesis of atherosclerosis. In this context, this receptor of the innate immune system has been shown to be of special relevance in hypercholesterolemia [13] and it has also been linked to atheroprotective effects of laminar, but not disturbed blood flow [14, 15]. We cannot change the blood flow and the architecture of vessels in humans to protect them from atherosclerosis, but we can try to change the blood through

pharmacology. In our study we demonstrated that ATV can control overexpression of proatherogenic endothelial TLR2 protein and TLR2-mediated endothelial activation. Moreover, ATV has cholesterol lowering and therefore additional atheroprotective properties.

Even with daily low-dose ATV a plasma concentration can be achieved that is beyond that which was active in our *in vitro* study. Thus, it is likely that these results may also be of relevance for the *in vivo* situation.

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