VIA MEDICA

ORIGINAL ARTICLE

Cardiology Journal
2012, Vol. 19, No. 1, pp. 20–28
10.5603/CJ.2012.0005

ISSN 1897–5593

Simvastatin and atorvastatin attenuate VCAM-1 and uPAR expression on human endothelial cells and platelet surface expression of CD40 ligand

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Abstract

Background: *In addition to their cholesterol lowering ability, statins have proven pleiotropic effects in the cardiovascular system. Chronic inflammation with interactions between platelets and endothelial cells leads to an upregulation of activity markers of atherosclerosis. The purpose of this study was to investigate the effects of simvastatin and atorvastatin on platelets and endothelial cells in an* in vitro *endothelial cell model.*

Methods and Results: *After a 24 h incubation period with either simvastatin or atorvastatin (1 μmol/L), human umbilical vein endothelial cells were stimulated for 1 h with lipopolysaccharide (LPS), and were then incubated in direct contact with activated platelets. Platelet surface expression of CD40L and CD62P and expression of ICAM-1, VCAM-1, uPAR and MT1-MMP on endothelial cells were measured by flow cytometry. Supernatants were analyzed by ELISA for soluble MMP-1. The increased expression of VCAM-1 and uPAR on endothelial cells by stimulation with LPS and by direct contact with activated platelets was significantly reduced to a similar extent through pre-incubation with both atorvastatin and simvastatin ($p < 0.05$). Platelets without endothelial cell contact, but in direct contact with either statin, showed similar significant reductions in surface expression of CD40L ($p < 0.005$).*

Conclusions: *These effects may explain the ability of statins to reduce the progression of atherosclerosis in addition to their cholesterol-lowering properties.* (Cardiol J 2012; 19, 1: 20–28)

Key words: platelets, endothelial cells, simvastatin, atorvastatin, atherosclerosis

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Received: 23.06.2011 Accepted: 12.09.2011

Introduction

The pathogenesis of atherosclerosis, the leading cause of morbidity and mortality in industrial countries, is multifactorial. Cardiovascular (CV) risk factors such as hypertension, diabetes, cigarette smoking, family history and elevated serum lipid levels contribute to the initiation and progression of atherosclerosis [1–5]. Interactions between platelets and endothelium play an important role in the pathogenesis of atherosclerosis and CV diseases, leading to severe clinical events such as myocardial infarction (MI) and stroke. It is characterized by the formation of plaque consisting of foam cells, immune cells, vascular endothelial cells, smooth muscle cells, platelets, extracellular matrix and a lipid-rich core [2, 6, 7]. Moreover, interactions between platelets and endothelial cells mediate essential processes in the development of atherosclerosis by an increased expression of vascular cell adhesion molecules and their ligands [8–12]. CD62P and CD40L are expressed on activated platelets and are directly involved in the interaction of platelets with leukocytes and endothelial cells [13]. Binding of CD40L to endothelial CD40, which is the counter-receptor for platelet-derived CD40L, leads to the release of interleukin-8, tissue factor and MCP-1, the major chemoattractants for neutrophils and monocytes [14–16]. In addition, activation of CD40 on endothelial cells leads to increased expression of various endothelial adhesion receptors such as E-selectin, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 [17–19]. Upregulation of endothelial VCAM-1 and ICAM-1 expression increases vulnerability of the atherosclerotic vascular plaque to fissuring rupture and superimposed thrombosis, leading to the clinical scenario of unstable angina, acute MI or sudden cardiac death [20–22]. Engagement of the endothelial CD40 receptor and platelet CD40L results in an increased production of inflammatory cytokines, adhesion molecules and matrix-degrading proteases (MMPs). MMPs degrade various proteins of the extracellular matrix and promote inflammation and the destruction of the inflamed tissue. Additionally, imbalance in the plasminogen and matrix metalloproteinase activation systems may lead to destabilization of the vulnerable fibrous cap of the atherosclerotic plaque [23–25].

Statins are highly effective lipid-lowering agents, widely used in medical practice [26]. Simvastatin and atorvastatin are representative of this class and are commonly prescribed and applied statins. Hypercholesterolemia impairs endothelial function and, by blocking 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), statins inhibit an early rate-limiting step in cholesterol biosynthesis [27]. Furthermore, statins decrease smooth muscle cell migration and proliferation [28], decrease pre-pro endothelin-1 (ET-1) mRNA expression and increase basal nitric oxide dilator functions of the endothelium [29]. Because of the strong association between elevated serum cholesterol levels and coronary atherosclerotic disease, it has been suggested that the reduction of serum cholesterol levels by statins is the main mechanism underlying their beneficial effects [30].

However, recent evidence suggests that statins have pleiotropic effects, independent of lipid balance regulation, and that this may partially explain their role in decreasing CV mortality and morbidity. The influence of statin treatment on various soluble cellular adhesion molecules (CAMs), including ICAM-1, has already been described in hypercholesterolemic patients [31]. Pleiotropic statin effects may be due to an attenuation of interactions between platelets and vascular endothelial cells and the release and expression of activity markers of atherosclerosis. Therefore, the purpose of the present study was to investigate the effects of simvastatin and atorvastatin on platelets and endothelial cells in an *in vitro* endothelial cell model.

Methods

Coincubation of simvastatin or atorvastatin pre-incubated endothelial cells with platelets

Human umbilical vein endothelial cells (HUVEC) were prepared as previously described [32, 33] and cultured in endothelial cell basal medium (PromoCell) containing 2% fetal calf serum (FCS); 1 μg/mL hydrocortisone (HC-500); 0.4% endothelial cell growth supplement (ECGS/H-2); 0.1 ng/mL epidermal growth factor (hEGF-0.05); 1 ng/mL basic fibroblast growth factor (hbFGF-0.5); 5 ng/mL amphotericin and 50 μg/mL gentamicin. The confluenced endothelial cells were added to 12-well plates and incubated for 24 h with 1 mmol/L nicotinic acid (Merck, Hohenbrunn, Germany). Platelets were prepared from the blood of healthy probands as described [34, 35]. Washed platelets were stimulated for 30 min with thrombin (0.5 U/mL) and lipopolysaccharide (LPS) (1,000 ng/mL). Before coincubation experiments, thrombin activity was antagonized by hirudin (5 U/mL). Pretreated platelets (final concentration 2×10^8/mL) were added to confluent endothelial monolayers with and without nicotinic acid. After 60-min coincubation under

cell culture conditions, all platelets were removed by gentle washing, which was confirmed by light microscopy. After six more hours of incubation of the endothelial cells, the supernatant was aspirated, centrifuged at 2,000 g and stored at –80°C [36]. Following this incubation, the expression of activity markers on platelets, as well as that on endothelial cells, was measured by flow cytometry.

Flow cytometric analysis

Flow cytometric analysis of platelets and endothelial cells was performed by gating in forward and side scatter. Platelets were gated back for determination of the expression of CD40L and CD62P. For the analysis of platelets, 100 μL of each sample were stained for 15 min at room temperature with 10 μL aliquots of mouse anti-human CD40L-FITC antibodies (BD Pharmingen, Heidelberg, Germany) and mouse anti-human CD62P-PE antibodies (Beckman-Coulter, Krefeld, Germany). Endothelial cells were gated back for determination of the surface expression of ICAM-1, VCAM-1, urokinase receptor uPAR and membrane type 1 matrix metalloprotease (MT1-MMP). For the analysis of endothelial cells, 100 μL of each sample were stained for 15 min at room temperature with 10 μL aliquots of anti-human CD54 PE-Cy5 (ICAM-1 from BD Pharmingen, Heidelberg, Germany), anti-human CD106-FITC (VCAM-1 from R&D Systems, Inc., Wiesbaden, Germany), anti-human CD87-FITC (uPAR from American Diagnostica Inc., Stamford, CT, USA), anti-human MT1-MMP (Ab-1) Mouse mAb (114-IF2) (Merck Chemicals Ltd., Nottingham, UK). Corresponding isotypes (Beckman Coulter, Marseille, France) were used as a control. All flow cytometric analyses were performed on an EPICS XL-MCL analyzer (Beckman Coulter, Krefeld, Germany) equipped with an argon laser tuned at 488 nm. Mean fluorescence intensity was measured and all FACS data is expressed as MFI in this manuscript. System II Version 3.0 software was used for data acquisition and evaluation. Compensation of the four channel fluorescence was adjusted for precision using Cyto-CompTM reagents and Cyto-TrolTM control cells (Coulter Immunotech, Krefeld, Germany).

Enzyme linked immunosorbent assay (ELISA)

The concentration of MMP-1 (Human, Biotrak ELISA System, GE Healthcare Ltd, UK) in the supernatants was determined by sandwich-type immunoassay following the manufacturer's instructions. All concentration analyses were performed on an ELISA-Reader — Lab Systems Multiskan RC (Labsystems, Finland). Genesis Lite Software, ELISA Multiskan RC was used for data acquisition and evaluation.

Statistical analysis

All calculations were performed using SAS release 9.2 (SAS Institute Inc. Cary, NC, USA). Numerical data was expressed as means ± standard deviation. A Dunnett's test was applied as parametric test. A two-tailed probability value < 0.05 was considered significant.

The study was approved by the local bioethical committee and all patients gave their informed consent.

Results

Effects of simvastatin and atorvastatin on endothelial cell surface markers

HUVEC pre-incubation with simvastatin resulted in significantly decreased surface expression of VCAM-1 from 1.37 ± 0.57 to 0.69 ± 0.05 ($p = = 0.013$) after contact with resting platelets, and from 1.26 ± 0.4 to 0.69 ± 0.07 ($p = 0.007$) after contact with thrombin-stimulated platelets. Pre-incubation with simvastatin significantly reduced surface expression of VCAM-1 from 1.4 ± 0.6 to 0.66 ± ± 0.05 ($p = 0.014$) after contact with LPS-stimulated platelets, and from 1.4 ± 0.66 to 0.7 ± 0.1 ($p = 0.03$) after contact with LPS- and thrombin--stimulated platelets (Fig. 1).

HUVEC pre-incubation with simvastatin resulted in significantly decreased surface expression of uPAR from 0.4 ± 0.1 to 0.27 ± 0.04 ($p = 0.038$) after contact with platelets, and from 0.56 ± 0.17 to 0.33 ± 0.06 ($p = 0.035$) after contact with thrombin-stimulated platelets. Pre-incubation with simvastatin significantly reduced surface expression of uPAR from 0.46 ± 0.13 to 0.27 ± 0.04 ($p = 0.015$) after contact with LPS-stimulated platelets (Fig. 2).

HUVEC pre-incubation with atorvastatin resulted in significantly decreased surface expression of VCAM-1 from 1.37 ± 0.57 to 0.7 ± 0.2 ($p = 0.01$) after contact with platelets, and from 1.26 ± 0.4 to 0.68 ± 0.12 ($p = 0.007$) after contact with thrombin--stimulated platelets. Pre-incubation with atorvastatin significantly reduced surface expression of VCAM-1 from 1.4 ± 0.6 to 0.57 ± 0.12 ($p = 0.008$) after contact with LPS-stimulated platelets, and from 1.4 ± 0.66 to 0.55 ± 0.18 ($p = 0.01$) after contact with LPS- and thrombin-stimulated platelets (Fig. 1).

HUVEC pre-incubation with atorvastatin resulted in significantly decreased surface expression

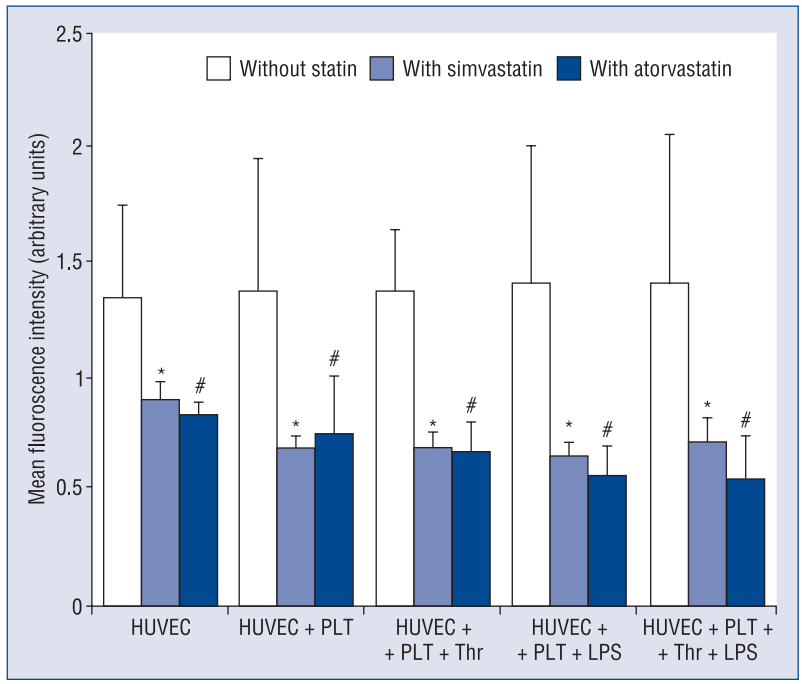

Figure 1. Surface expression of VCAM-1 on HUVEC with and without 24 h pre-incubation with simvastatin or atorvastatin; N = 7 experiments; PLT — platelets; Thr — thrombin; LPS — lipopolysaccharide; *p < 0.05 against not pre-treated HUVEC; #p < 0.05 against not pre-treated HUVEC.

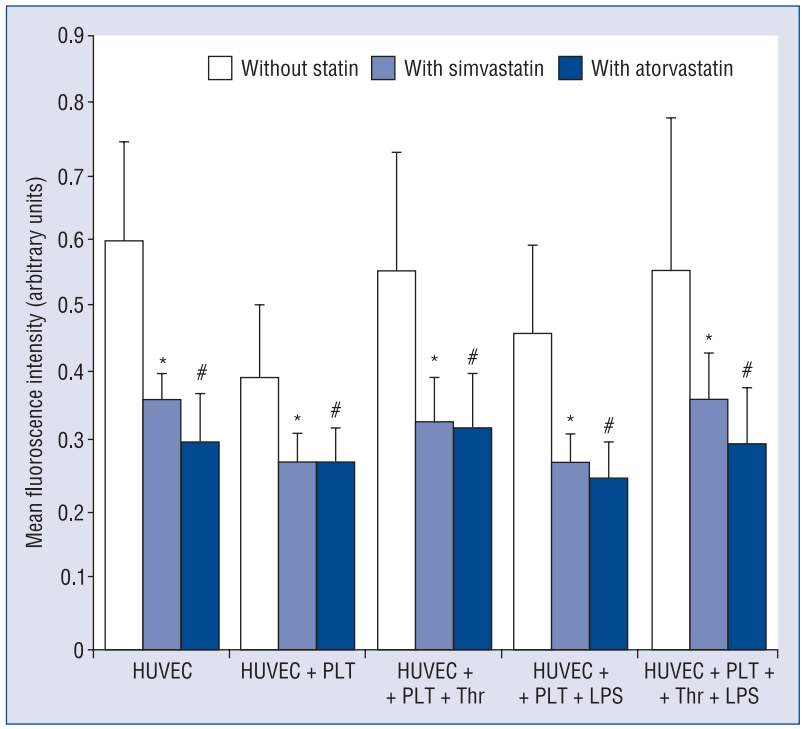

Figure 2. Surface expression of uPAR on HUVEC with and without 24 h pre-incubation with simvastatin or atorvastatin; N = 7 experiments; PLT — platelets; Thr — thrombin; LPS — lipopolysaccharide; *p < 0.05 against not pre-treated HUVEC; #p < 0.05 against not pre-treated HUVEC.

of uPAR from 0.4 ± 0.1 to 0.27 ± 0.04 (p = 0.03) after contact with platelets, and from 0.56 ± 0.17 to 0.32 ± 0.079 (p = 0.02) after contact with thrombin-stimulated platelets. Pre-incubation with atorvastatin significantly reduced surface expression of uPAR from 0.46 ± 0.13 to 0.25 ± 0.05 (p = 0.007) after contact with LPS-stimulated platelets, and from 0.55 ± 0.22 to 0.3 ± 0.08 (p = 0.03) after contact with LPS- and thrombin-stimulated platelets (Fig. 2).

Regarding ICAM-1 expression on HUVEC, pre-incubation with simvastatin significantly increased surface expression of ICAM-1 from 21.03 ± ± 10.7 to 67.44 ± 11.5 (p = 0.0051) after contact with platelets, and from 26.5 ± 14.2 to 52.32 ± ± 20.25 (p = 0.049) after contact with thrombin-stimulated platelets (Fig. 3). Additionally, pre-incubation with atorvastatin significantly increased surface expression of ICAM-1 from 21.03 ± 10.7 to 76 ± 25.8 (p = 0.0018) after contact with platelets, and from 57.3 ± 25.9 to 133.7 ± 50.9 (p = 0.02) after contact with LPS-stimulated platelets (Fig. 3).

Simvastatin or atorvastatin had no significant effects on the surface expression of MT1-MMP on endothelial cells.

In a comparison between simvastatin and atorvastatin, we also observed no significant differences in their effects on human umbilical vein endothelial cell surface markers.

Endothelial cell mediated and direct effects of simvastatin and atorvastatin on platelets

Platelet surface expression of CD62P did not differ significantly between platelets in direct contact with simvastatin or atorvastatin pre-incubated endothelial cells compared to platelets incubated with untreated endothelial cells (Fig. 4). Surface expression of CD40L on platelets after one hour's direct contact with HUVECs, pre-incubated with simvastatin, and stimulated with LPS, significantly decreased from 1.99 ± 0.09 to 1.66 ± 0.26 (p = = 0.0038) (Fig. 4). To discriminate between those effects mediated by pre-treated endothelial cells and the possible direct effects of simvastatin or atorvastatin on platelets, platelets were directly incubated with either simvastatin or atorvastatin solely for 1 h. When this was done, simvastatin incubation significantly increased CD62P expression on unstimulated platelets from 0.99 ± 0.1 to 1.31 ± 0.13 (p = 0.0012), and atorvastatin incubation significantly increased CD62P expression on unstimulated platelets from 0.99 ± 0.1 to 1.21 ± 0.12 (p = 0.01), and when stimulated with LPS and thrombin from 4.43 ± 3.14 to 6.78 ± 0.7 (p = 0.0047) (Fig. 5). Surface expression of CD40L on platelets under stimulation with LPS was significantly reduced by simvastatin from 2.14 ± 0.19

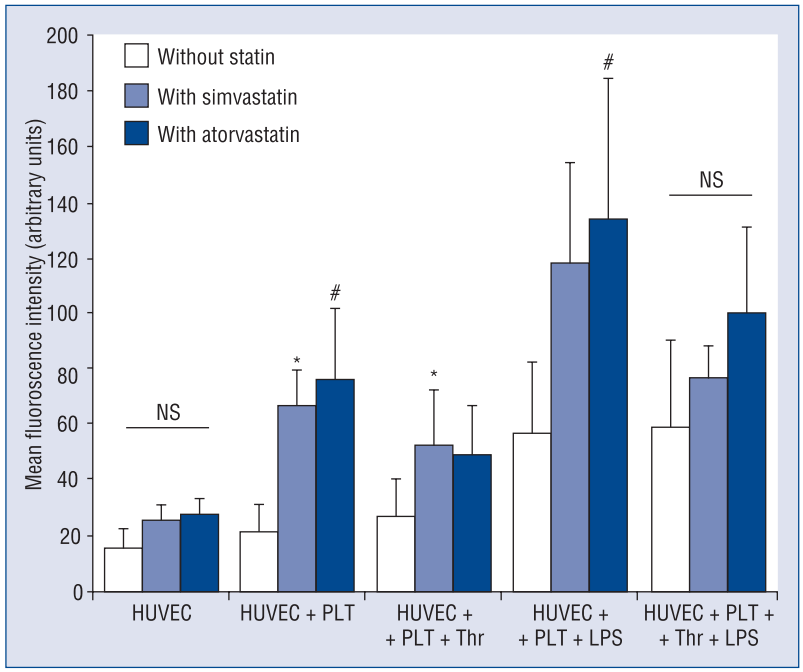

Figure 3. Surface expression of ICAM-1 on HUVEC with and without 24 h pre-incubation with simvastatin or atorvastatin; N = 7 experiments; PLT — platelets; Thr — thrombin; LPS — lipopolysaccharide; NS — not significant; *p < 0.05 against not pre-treated HUVEC; #p < 0.05 against not pre-treated HUVEC.

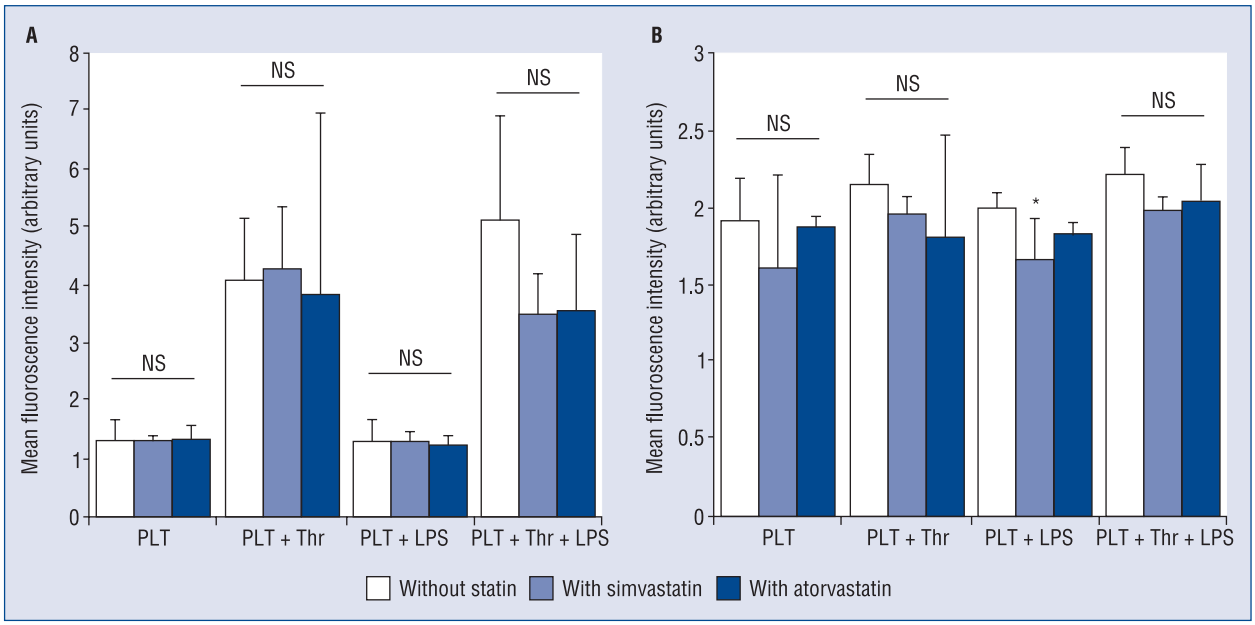

Figure 4. Surface expression of CD62P (**A**) and CD40L (**B**) on platelets after 1 h of direct contact with HUVECs, with and without pre-incubation with simvastatin or atorvastatin; N = 7 experiments; PLT — platelets; Thr — thrombin; LPS — lipopolysaccharide; NS — not significant; *p < 0.05 *vs* non pre-treated platelets.

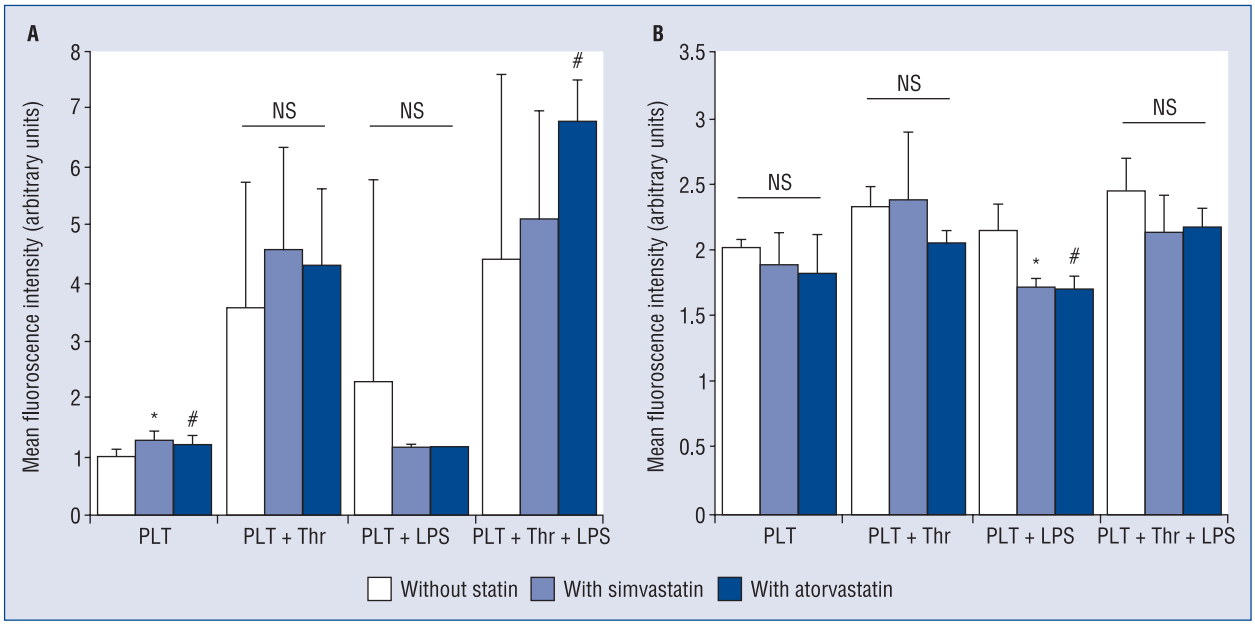

Figure 5. Direct effects of simvastatin or atorvastatin on surface expression of CD62P (**A**) and CD40L (**B**) on platelets. Platelets were directly incubated with simvastatin or atorvastatin for 1 h and stimulated with lipopolysaccharide (LPS) and/or thrombin (Thr); N = 7 experiments; PLT — platelets; NS — not significant; *p < 0.05 *vs* non pre-treated platelets; #p < 0.05 *vs* non pre-treated platelets.

to 1.72 ± 0.06 (p = 0.0002) and by atorvastatin from 2.14 ± 0.19 to 1.69 ± 0.11 (p < 0.0001) (Fig. 5).

In a comparison between simvastatin and atorvastatin, we observed no significant differences in their individual effects on platelet surface markers.

Effects of simvastatin and atorvastatin on HUVEC supernatant levels of soluble MMP-1

Upon direct HUVEC stimulation with interleukin 1β, supernatant levels of MMP-1 were lower on HUVEC pre-treated with simvastatin (1.97 ±

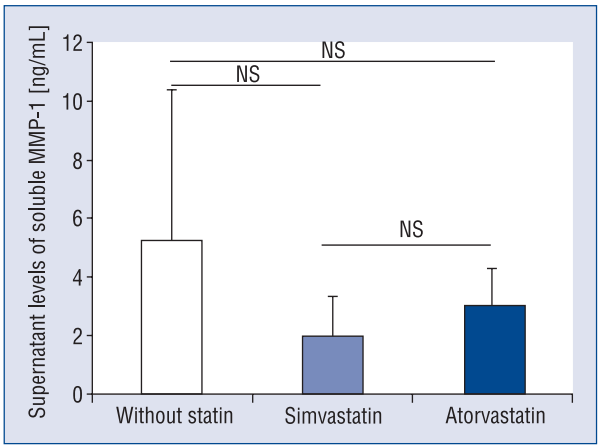

Figure 6. Supernatant levels of soluble MMP-1 upon stimulation with interleukin 1β on HUVEC with and without pre-incubation with simvastatin or atorvastatin; NS — not significant.

± 1.38 ng/mL; p = 0.39) and with atorvastatin (3.06 ± 1.23 ng/mL; p = 0.55) compared to untreated HUVEC (5.22 ± 5.15 ng/mL), although these differences were not statistically significant (Fig. 6).

Discussion

Many clinical studies, as well as data from recent experimental studies, have revealed that statins have additional effects beyond their serum cholesterol-lowering properties and that statins modify various biological processes in the vessel wall [12, 28–31, 37–43]. In the present study, simvastatin and atorvastatin reduced the expression of pro-atherogenic activity and progression markers on platelets and endothelial cells under pro-inflammatory conditions in an *in vitro* endothelial cell model. Simvastatin and atorvastatin both significantly reduced the expression of VCAM-1 and uPAR on endothelial cells after direct contact with activated platelets and surface expression of CD40L on platelets. These results underpin the extensive atheroprotective effects of statins, which may, in addition to their lipid-lowering potential, contribute to the inhibition of atherosclerosis progression.

The benefit of statin therapy appears to exceed the cholesterol-lowering effect by blocking HMG-CoA reductase, possibly by protective effects on endothelial nitric oxide bioactivity [29] and atherosclerotic plaque stabilization [28, 40, 41]. In addition, a recent study has shown that statins can suppress inflammatory response independently of HMG-CoA reductase inhibition by means of binding directly to a novel regulatory site of the β_2 integrin via inhibiting leukocyte function antigen-1 [37]. The mechanism of anti-inflammatory properties of statins was further described by Yoshida et al. [38], who demonstrated that cerivastatin reduced monocyte adhesion to vascular endothelium by decreasing expression of integrins and actin polymerization through inactivation of RhoA.

In 1990, Berk et al. [44] highlighted the role of inflammation in coronary artery disease with an elevation of C-reactive protein (CRP). In the CARE trial, statins significantly decreased plasma hs-CRP levels over a five-year period in patients who did not experience recurrent coronary events. This emphasizes the direct anti-inflammatory potential of statins [39]. These studies indicate that statins are effective in decreasing systemic and vascular inflammation. The CD40-CD40L signalling pathway plays a key role in the modulation of inflammatory responses between vascular cells and platelets, involving adhesion molecules, pro-inflammatory cytokines and chemokines. Statins have been proven to decrease CD40 expression and CD40-related activation of vascular cells [40, 41]. Mosheimer et al. [43] showed that activated platelets induce COX-2 expression in HUVEC, and that this effect can be reversed by pre-incubation of platelets with atorvastatin; this effect is due to the down-regulation of CD40L on activated platelets by atorvastatin. It is worth noting that in the present study we were able to support these previous findings: simvastatin and atorvastatin induced a significant reduction of CD40L expression on platelets, whereas no significant individual differences between both statins regarding CD40L platelet surface expression could be observed. The simvastatin and atorvastatin *in vitro* concentration applied in the present study was 1 μmol/L, which is within the range of effective serum concentrations seen in clinical practice [42, 45].

A limited number of randomized controlled clinical trials comparing the use of atorvastatin to that of simvastatin have shown that atorvastatin is more effective in reducing total cholesterol levels and LDL-cholesterol levels, as well as the rate of CV events [46–48]. Jacobson et al. [49] found in a retrospective study that the risk of the first CV event was significantly lower among patients in whom atorvastatin, rather than simvastatin, had been newly initiated. uPAR is present at increased levels in atherosclerosis, and correlates directly with CV disease severity [50].

In our study, atorvastatin and simvastatin both significantly reduced the expression of VCAM-1 and uPAR on endothelial cells and CD40L surface expression on platelets. However, we observed no

significant differences between the statins regarding individual effects on human umbilical vein endothelial cell and platelet surface markers. In the ARMYDA-ACS study, Patti et al. [51] demonstrated an increased expression of ICAM-1 and VCAM-1 after preloading with high dose atorvastatin and after percutaneous coronary intervention. A possible explanation for the increased expression of ICAM-1 may be the periprocedural use of glycoprotein IIb/IIIa inhibitors. Our study also showed a significantly increased expression of ICAM-1 on endothelial cells preincubated with statins and after stimulation with activated platelets. However, we cannot provide detailed mechanistic insights as to why statins increased the expression of ICAM-1 on endothelial cells, but significantly decreased VCAM-1 effects. Future studies will be needed to further explore the possible mechanism which supports the influence of statins on CV diseases.

Conclusions

We demonstrated that simvastatin and atorvastatin both extenuate the expression of proatherogenic markers on endothelial cells and platelets under pro-inflammatory conditions. These effects may explain the ability of statins to reduce CV events and progression of atherosclerosis in addition to their cholesterol-lowering properties.

Conflict of interest: none declared

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