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Atorvastatin induces tissue transglutaminase in human endothelial cells ☆

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Abstract

Tissue transglutaminase (tTgase) contributes to the organisation of the basement membrane and is therefore thought to be important for the integrity and stability of the vessel wall. In the present study, we hypothesised that the HMG-CoA reductase inhibitor atorvastatin may up-regulate the tTgase expression in endothelial cells and thereby exert beneficial effects on endothelial function. Treatment of human umbilical vein endothelial cells (HUVEC) with atorvastatin (1–10 μM) caused a clear increased expression of tTgase in both permeabilised and non-permeabilised HUVEC. In contrast, stimulation of HUVEC with TNFα had no substantial effect on tTgase expression or localisation but inhibited the atorvastatin-induced up-regulation and externalisation of tTgase. Propidium iodide staining revealed that statin-induced apoptosis is not responsible for the enhanced expression. By inducing the expression of tTgase, statins may promote tTgase-mediated stabilisation of the basement membrane. This effect of atorvastatin may contribute to the beneficial role of statins on endothelial function.

Keywords: Tissue transglutaminase; Endothelial cells; Extracellular matrix; Atherosclerosis; Coronary heart disease

Endothelial cells, apart from forming the barrier between the bloodstream and the vessel wall, have also influence on vessel structure, permeability, vessel tonus, platelet function, haemostasis, and inflammation [1,2]. The integrity of the endothelium is regulated on different levels such as intercellular contacts, adhesion molecules, basement membrane, and extracellular matrix. The extracellular matrix is an important determinant of plaque stability in acute coronary syndromes. Mechanical forces and matrix metalloproteinase activity initiate plaque rupture, whereas tissue inhibitors of metalloproteinases have an important albeit indirect role in plaque stabilisation.

Tissue transglutaminase (tTgase), a calcium-dependent enzyme that catalyses the formation of epsilon (γ-glutamyl)lysine isopeptide bonds that are resistant to enzymatic, mechanical, and chemical degradation, is thought to stabilise the plaque directly. In particular, tTgase has been shown to cross-link components of the extracellular matrix, including fibronectin, vitronectin, laminin, and collagen [3–6]. Therefore, tTgase activity plays an important role in stabilisation of the basement membrane and adhesion of cells [7], which are important processes in wound healing, angiogenesis, and bone remodelling [8–10]. The expression of tTgase was documented in a variety of cell types, including endothelial cells, smooth muscle cells, and macrophages [11,12], which are major components of atherosclerotic lesions. Moreover, tTgase was found to be highly expressed along the luminal endothelium and in smooth muscle cells in coronary and carotid artery plaques [13]. At the sites of

☆ *Abbreviations:* tTgase, tissue transglutaminase; EC, endothelial cells; SMC, smooth muscle cells; HUVEC, human umbilical vein endothelial cells; TNFα, tumor necrosis factor α.

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neovascularisation and the fibrous cap, the expression of tTGase is high, while the enzyme is weakly expressed at the lipid core and in accumulating macrophages, suggesting that decreased expression of tTgase might contribute to the destabilisation of the atherosclerotic plaque [13].

HMG-CoA-reductase inhibitors (statins) were shown to have anti-atherogenic effects on different levels. Clinical trials have consistently shown that statins have many pleiotropic effects beyond lipid lowering. A subgroup analysis of the West of Scotland Coronary Prevention Study (WOSCOPS) [14] showed that cholesterol-independent mechanisms may provide additional benefits. Other studies as the Scandinavian Simvastatin Survival Study (4S) [15] and the Heart Protection Study (HPS) [16] point into the same direction. In vitro studies have shown positive effects of statins on the improvement of the endothelial dysfunction, reduction of inflammatory response, stabilisation of the atherosclerotic plaques and reduction of thrombogenic response [17].

As up-regulation of tTgase in endothelial cells with consecutive stabilisation of the atherosclerotic plaque would promise a clinical benefit we investigated if statin inhibitors up-regulate tTgase expression in human endothelial cells.

Methods

Cell culture. Cell culture of human umbilical vein endothelial cells (HUVECs) was performed as described previously [18]. In brief, the umbilical vein was incubated with 0.1% dispase, HUVEC were washed out and cultured in endothelial basal medium with endothelial cell growth supplement (PromoCell, Heidelberg, Germany). The cells were treated with the atorvastatin at final concentrations of 0.1–10 μM for 24, 48, or 72 h. Afterwards, the cells were detached with accutase and subsequently used for FACS and Western blot analysis.

Tumor necrosis factor α (TNFα) was purchased from Biomol (Hamburg, Germany), atorvastatin was a kind gift from Parke–Davis, Ann Arbor (USA).

Flow cytometry. For detection of tTgase in HUVEC the endothelial cells were permeabilised by Dako Intra Stain (1:100, DakoCytomation, Hamburg, Germany). Unspecific binding was blocked by incubation with 0.5% BSA in PBS for 30 min at 4 °C. Afterwards the cells were treated with a mouse monoclonal anti-tTgase antibody (CUB 7402, DakoCytomation) and a FITC-labelled secondary antibody (Sigma, Munich, Germany). After staining cells were immediately assayed in Becton–Dickinson FACS Calibur (Becton–Dickinson, Heidelberg, Germany). Staining of surface tTgase was performed in an analogous manner without permeabilisation. Results are expressed as mean fluorescence intensity (MFI).

Apoptosis in HUVEC was assayed by Propidium Iodide (PI, Sigma) staining. Therefore, detached HUVEC were incubated with 1 μg/mL PI for 30 min. Depending on the cell cycle PI would be incorporated into the DNA. The fluorescence of the HUVEC reflecting the cell cycle was analysed using the FACS Calibur.

Western blot. After treatment of HUVEC with atorvastatin, the cells were washed with PBS and lysed with 0.1 mL/10^6 cells of lysis buffer (50 mM Hepes, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 1.9 mg/mL aprotinin, and 0.5 mg/mL leupeptin, pH 7.4). After having assayed the protein concentration the proteins (30 μg per lane) were separated by 10% SDS–PAGE under reducing conditions and transferred to a PVDF membrane. Non-specific binding was blocked by 10% dried milk in TBS–Tween (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20, pH 7.5) overnight at 4 °C. Incubation with anti-tTgase monoclonal antibody (1:100) was followed by incubation with horseradish peroxidase-conjugated goat anti-mouse antibody (1:5000). Staining was visualised by enhanced chemiluminescence system (Amersham, Buckinghamshire, UK). To confirm equal protein loading membranes were stained with Ponceau S.

Statistics. Data are presented as means ± SD. Statistical significance was calculated using Student's t test for paired samples. A value of $p < 0.05$ was considered significant.

Results

Atorvastatin induces tTgase expression in EC in a dose- and time-dependent manner

HUVEC were treated with atorvastatin at doses ranging from 0.1 to 10 μM over 24, 48 or 72 h. The surface expression of tTgase was then measured after staining with a monoclonal primary antibody and a FITC-conjugated secondary antibody. FACS analysis revealed that atorvastatin induces dose-dependently tTgase expression in HUVEC (Figs. 1A–C). Independent of the incubation time, this effect was most pronounced at 10 μM. Induction of tTgase by atorvastatin was detectable after 24 h, but significantly more pronounced after 48 and 72 h of atorvastatin treatment.

To distinguish between an enhanced intracellular storage and surface-bound tTgase we performed FACS analysis with permeabilised HUVEC. Again we found a significantly increased staining for tTgase in the HUVEC after atorvastatin treatment (Fig. 1D). Enhanced externalisation was also time- and dose-dependent (data not shown). Compared to surface staining of tTgase we found a higher basal expression in the cytoplasm reflecting the fact that most tTgase is stored intracellularly.

Western blot analysis of whole cell lysate confirmed that atorvastatin (48 h treatment) dose-dependently increased the expression of tTgase protein in HUVEC (Fig. 2). Again the effect was most pronounced at 10 μM.

Atorvastatin-induced up-regulation of tTgase in EC is not caused by increased cell apoptosis

As it is known that enhanced tTgase expression is related to apoptosis, we wanted to exclude atorvastatin-induced apoptosis as a possible cause of up-regulation of tTgase. Therefore, we performed propidium iodide staining of atorvastatin-treated HUVEC. Atorvastatin concentrations up to 9 μM did not induce significant cell apoptosis, whereas 10 μM atorvastatin significantly enhanced apoptosis of HUVEC (Fig. 3).

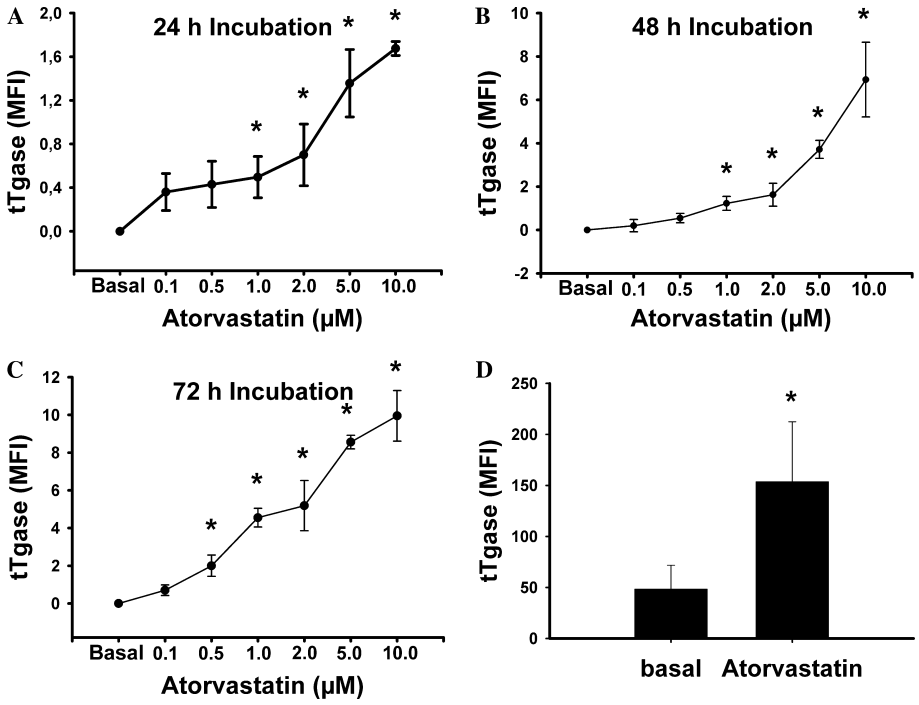

Fig. 1. Up-regulation of tTgase expression in HUVEC after treatment with atorvastatin. (A–C) HUVEC were treated with 0.1–10 μM atorvastatin over 24 h (A), 48 h (B) or 72 h (C). Ordinate represents the isotype-corrected MFI for tTgase fluorescence. (D) HUVEC treated with 2 μM atorvastatin for 48 h. Expression of tTgase was analysed in permeabilised HUVEC in comparison to untreated cells. Values are expressed as means ± SD, $n = 3$, $^*p < 0.05$.

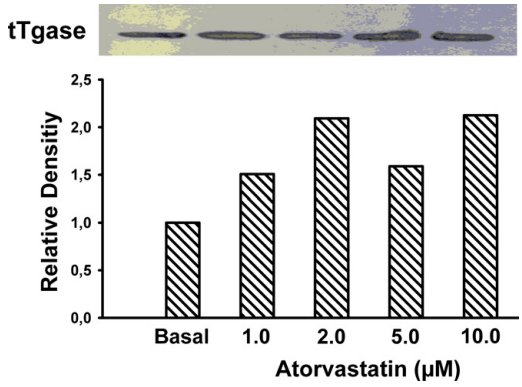

Fig. 2. Atorvastatin enhances tTgase expression in whole cells lysate of HUVEC. HUVEC were treated with different concentrations of atorvastatin for 48 h and after lysis analysed by Western blot. Bands are representative for three independent experiments. In densitometric analysis basal density was set to 1.

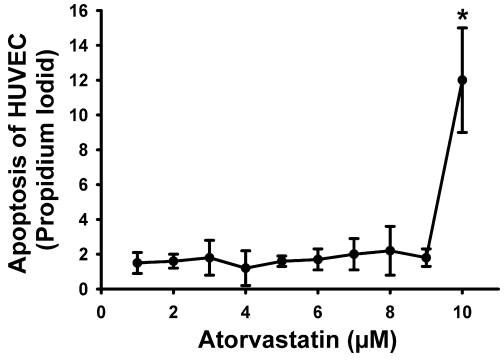

Fig. 3. Atorvastatin-induced up-regulation of tTgase in HUVEC is not caused by increased apoptosis. HUVEC were incubated with different concentrations of atorvastatin for 48 h and then stained with PI. Ordinate gives MFI for PI fluorescence. Values are given as means ± SD, $n = 3$, $^*p < 0.05$.

Atorvastatin-induced up-regulation of tTgase in EC is inhibited by TNF-α

Since atorvastatin treatment at 10 μM resulted in enhanced cell apoptosis, lower concentrations of statins were used in further experiments. Whereas atorvastatin (5 μM, 48 h of incubation) significantly up-regulated tTgase in HUVEC, TNF (250 U/mL) did not (Fig. 4). Simultaneous supplementation of atorvastatin and TNFα to HUVEC, however, prevented atorvastatin-induced up-regulation of tTgase ($p < 0.05$).

Discussion

This study shows for the first time an enhanced production and externalisation of tTgase in HUVEC by incubation with the HMG-CoA reductase inhibitor

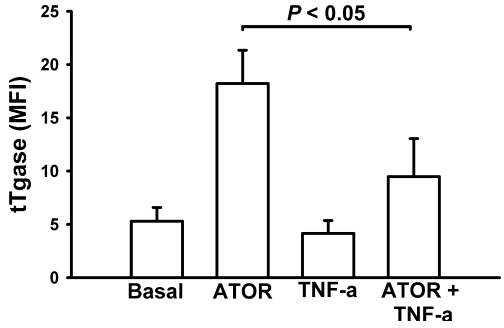

Fig. 4. TNFα inhibits atorvastatin-induced up-regulation of tTgase expression in HUVEC. HUVEC were incubated with atorvastatin (5 μM, 48 h), TNFα (250 U/mL, 48 h) or both. Values are given as means ± SD, $n = 3$, $^*p < 0.05$.

atorvastatin. The induction was found to be dose- and time-dependent. Over-expression of tTgase in other cell types such as fibroblasts results in increased cell spreading and reduced susceptibility to detachment with trypsin [19]. Moreover, enhancement of tTgase expression by thrombin has been linked to improved plaque stability [20]. On the other hand, reduced expression of this enzyme leads to diminished cell adhesion and spreading of endothelial cells [7].

The localisation of tTgase is primarily cytoplasmic, with a small part of its intracellular pool present in the nucleus [21]. In addition, some amounts of the enzyme are present on the cell surface and in the extracellular matrix [22,23]. We found an atorvastatin-dependent up-regulation both in the cytoplasm and on the endothelial cell surface. While intracellular expression of tTgase is related to the programmed cell death, the extracellular expression is regarded as an important factor in cell adhesion and matrix stabilisation. Surface-bound tTgase has been shown to interact with 1 and 3 integrins and to potentiate integrin signalling [24]. It also colocalises with 1 integrin in endothelial cells [25], which is known as an important protein both for efficient cell spreading and adhesion. Additionally, the surface bound form is important in cross-linking of extracellular matrix products.

It has been reported that statins induce apoptosis in vascular endothelial cells. This effect is thought to be specific for hydrophobic statins such as atorvastatin, lovastatin, and simvastatin. It was shown to be a dose-dependent effect, with apoptosis occurring at concentrations above 1 μM. Kaneta et al. [26] demonstrated decreased endothelial cell viability at 7.9 ± 0.1 μM atorvastatin. The effect was clearly dependent on geranylgeranylpyrophosphate and farnesylpyrophosphate, indicating a Rho A involvement [26,27]. Induction of tTgase occurs during apoptosis, in fact, tTgase mRNA is transcribed as a consequence of apoptosis induction. Over-expression of tTgase in many cell lines enhances their susceptibility to apoptosis, indicating a pivotal role for tTgase in this process [28]. Therefore, we investigated if atorvastatin-induced apoptosis is the mechanism involved in the induced tTgase expression. We found no induction of apoptosis in HUVEC analysed after atorvastatin treatment up to a concentration of 9 μM. However, higher doses of atorvastatin induced apoptosis in HUVEC, indicating that the tTgase induction by 10 μM atorvastatin could at least in part be due to enhanced apoptosis.

There are controversial reports about the induction of tTgase by TNFα. Kim et al. [29] reported that TNFα has no influence on the tTgase expression, while Kuncio et al. [30] showed that TNFα results in up-regulation of the enzyme. In our experiments, however, we could not detect any significant influence of TNFα on the expression of surface bound tTgase. However, the fact that TNFα reduces the atorvastatin-induced up-regulation of tTgase, may be of importance.

In conclusion, by up-regulating the expression and by inducing the externalisation of this enzyme, statins may promote tTgase-mediated stabilization of the basement membrane of endothelial cells which may promote the integrity of the vessel wall. This unanticipated role of atorvastatin may contribute to the beneficial role of statins on endothelial function.

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