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Effect of statins on the proteasomal activity in mammalian endothelial and vascular smooth muscle cells

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Abstract

Inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, known as statins, effectively prevent cardiovascular events. In addition to their lipid lowering properties, a variety of pleiotropic effects on cardiovascular cells were demonstrated in vitro and in vivo. It has been hypothized that statins deploy a part of their effects by targeting the proteasome. Statin-induced effects remarkably overlap with effects obtained by inhibition of the proteasome in endothelial and vascular smooth muscle cells (e.g., endothelial nitric oxide synthase (eNOS)-upregulation, attenuation of nuclear factor kappa B (NF-κB) activation, inhibition of proliferation). We therefore examined, whether statins modulate the proteasomal activity of vascular cells. We studied the effect of simvastatin, atorvastatin, and pravastatin as well as of the proteasome inhibitor clasto-lactacystin on morphology, proliferation, viability, and proteasomal activity in two mammalian endothelial cell lines (CPAE and Ea.hy962), and in primary vascular smooth muscle cells (VSMCs). Both statins and lactacystin induced comparable morphological changes and attenuated proliferation of calf pulmonary artery cell line (CPAE). Whereas the statin-induced effects were reversed by mevalonic acid, however, the lactacystin-induced alterations were not influenced by mevalonic acid. As expected, lactacystin caused a significant loss of proteasomal activity measured in the extract of treated CPAE cells, whereas the extracts of statin-treated CPAEs exhibited unchanged activities. This result was also confirmed in Ea.hy926 cells and in primary rat VSMCs. We show here, that even high doses of statins do not modulate the activities of purified human 20S proteasomes. We conclude that the similar biological effects of statins and proteasome inhibitors in vascular cells are not due to a common inhibitory mechanism of action on the proteasome.

Keywords: Statins; Proteasome; Endothelial cells; Smooth muscle cells

1. Introduction

Statins are highly effective drugs, widely used in lipid lowering therapy. Their efficacy in preventing cardiovascular events has been demonstrated in several clinical trials for primary [1,2] and secondary prevention [3,4]. Nevertheless, results of in vitro studies and clinical trial analysis have revealed that – besides their lipid lowering properties – statins have various additional cholesterol-independent effects with respect to cardiovascular diseases [5]. These effects include improvement of endothelial function, reduction of pro-inflammatory events such as a decrease in monocyte adhesion and infiltration, and increased plaque stability [5–7]. Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), which catalyzes the conversion of 3-hydroxy-3-methylglutaryl-coenzyme A to mevalonic acid (MVA). These drugs consequently interfere with the major regulatory step in the pathway that leads to synthesis of cholesterol and a variety of important non-sterol isoprenoids, such as farnesylpyrophosphate and geranylgeranylpyrophosphate. These metabolites are required for posttranslational modification of proteins:

Abbreviations: CaspL, caspase-like activity; ChTL, chymotrypsin-like activity; DMSO, dimethylsulfoxide; eNOS, endothelial nitric oxide synthase; HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; MVA, mevalonic acid; NF-κB, nuclear factor kappa B; TL, trypsin-like activity; VSMC, vascular smooth muscle cell

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e.g., rho-GTPases. Inhibitors of HMGR potentially interfere with a variety of signaling pathways thereby exerting pleiotropic cellular effects [8]. A number of recent studies describe another property of statin drugs that possibly contributes to their pleiotropism: interaction with the ubiquitin–proteasome system [9–12].

The 20S proteasome is a complex enzyme formed from four stacked heptameric subunit rings, with three different, well-characterized hydrolytic activities: chymotrypsin-like (ChTL), trypsin-like (TL) and caspase-like (CaspL). In association with additional subunits of the PA700 activator, the 26S proteasome is formed, which is responsible for the degradation of ubiquitinated proteins [13]. It plays a major role in generation of antigenic peptides, in degradation of misfolded proteins, and in the regulated degradation of signal mediators and transcription factors [14,15]. Inhibition of proteasomal activities by specific inhibitors abrogates protein degradation and induces accumulation of cellular proteins, leading to cell cycle arrest, proliferation block and induction of apoptosis in numerous cell types [16].

Studies on effects of statins on the proteasome are spare and contradictory. Because both the statin pro-drugs (lovastatin, mevastatin and simvastatin) and the selective proteasome inhibitor lactacystin (which undergoes lactonization in aqueous solution thereby forming clasto-lactacystin β-lactone) contain a β-lactone ring, demonstrated to be essential for lactacystin-mediated proteasome inhibition, it has been hypothized that statins deploy a part of their effects by targeting the proteasome [9]. Conflicting previous reports have described that statins inhibit, increase or have no effect on proteasomal activities, depending on the cell type or the assay systems used in these studies [9–12,17,18].

We succeeded in demonstrating that in vascular smooth muscle cells (VSMCs), inhibition of the proteasome by low doses of the specific inhibitor MG132 leads to reduced proliferation, and that high doses result in apoptosis of the cells [19,20]. Activation of nuclear factor kappa B (NF-κB) can be blocked by treatment of VSMCs with proteasome inhibitors administered according to concentration [19]. Proteasome inhibitors have been shown to significantly attenuate cytokine-stimulated leukocyte adhesion and endothelial cell adhesion molecule surface expression in human umbilical vein cells [21,22]. We have recently shown that low-dose proteasome inhibition enhances expression and activity of endothelial nitric oxide synthase (eNOS) in endothelial cells, and improves endothelial function of aortic rings [23].

The effects of proteasome inhibition remarkably overlap with pleiotropic effects obtained by the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in endothelial and vascular smooth muscle cells [24–26]. We therefore addressed the question, whether statins are able to modulate proteasomal activity of vascular endothelial cells and vascular smooth muscle cells.

2. Material and methods

2.1. Reagents

Simvastatin, in its β-lactone closed ring form (pro-drug), pravastatin, atorvastatin, lactacystin and mevalonic acid lactone were purchased from Sigma. Simvastatin and DL-mevalonic acid lactone were dissolved in ethanol, and atorvastatin, pravastatin and lactacystin were dissolved in dimethylsulfoxide (DMSO). Stocks were stored aliquoted at −80 °C.

2.2. Cell culture and treatment

Rat vascular smooth muscle cells were obtained from carotid arteries of male Wistar rats as described elsewhere [27].

Calf pulmonary artery cell line (CPAE) was purchased from the American Type Culture Collection. Cells were grown in MEM (Life Technologies, GIBCO) containing 10% FCS, 2 mM L-glutamine, 50 μg/ml streptomycin and 50 U/ml penicillin.

Cells of the line Ea.hy926, originally derived from human umbilical vein endothelial cells, were maintained in DMEM with penicillin (50 U/ml), streptomycin (50 mg/ml) and 10% fetal calf serum.

2.3. Assay of proteasomal activity

Proteasome chymotrypsin-like, trypsin-like and caspase-like activities from 20S proteasomes or cell lysates were determined fluorometrically in a spectramax GEMINI-EM (Molecular Devices) by using synthetic peptides linked to the fluorophor methylcoumarine. ChTL activity was measured by SLLVY-AMC hydrolysis, TL by BzVGR-AMC and CaspL activity by ZLLE-AMC hydrolysis with 360-nm excitation and 460-nm emission wavelengths.

Proteasomes (20S) were isolated from whole blood as described previously [28], and kept aliquoted at −80 °C. For activity measurements, 1 μg of the proteasome fraction was incubated in a volume of 100 μl 0.05 mM Tris buffer (pH 7.2) with tested drugs for 2.5 h at 37 °C. Then, fluorogenic substrates were added to a final concentration of 0.2 mM and further incubated for 30 min at 37 °C before measuring.

Cells were treated for 24 h with drugs or solvents as control. Cells were subsequently washed with PBS, and then scraped and lysed under hypotonic conditions with repeated cycles of thawing and freezing in liquid nitrogen. Lysates were centrifuged and the protein content of the supernatant was estimated by BCA protein assay (Pierce). Lysates were incubated for 30 min at 37 °C in incubation buffer containing an ATP-regenerating system (225 mM Tris–HCl, pH 8.2, 45 mM KCl, 7.5 mM $Mg(CH_3COO)_2$, 7.5 mM $MgCl_2$, 1.1 mM dithiothreitol, 6 mM ATP, 5 mM phosphocreatine, 0.2 unit of phosphocreatinekinase) and 0.2 mM of the appropriate fluorogenic substrate.

Enzymatic activity was normalized to protein concentration and expressed as a percentage of activity of solvent-treated control. The values are given as the means of three independent experiments ± S.E.M.

2.4. Assay of proliferation and viability

Adherent cells were counted in a hemocytometer 24 h after start of the treatment. Growth rate was calculated as net population doublings as described previously [29].

Cells were seeded in 96-well plates at 80% confluence (1×10^4 cells/well) and incubated in 2% FBS medium for 24 h. Substances were added at concentrations ranging from 1 to 50 μM. After 24 h, XTT was added to each well for 4 h. Formation of orange formazan was measured at 490 nm with an ELISA plate reader. Viability was expressed as percentage of solvent controls.

2.5. Fluorescence microscopy

Endothelial cells were treated with statins or lactacystin for 24 h. Cells were then fixed in 4% paraformaldehyde for 20 min, permeabilized in 1% Triton X-100, blocked in 1% serum and stained with Phalloidin-FITC for 1 h at 37 °C and afterwards with DAPI (Sigma, 1 μg/ml). Cells were photographed using an Axiovert S100 microscope (Zeiss) connected to a Sony MC-3254 video camera.

2.6. Western blotting

Cells were treated with statins or lactacystin for 24 h, washed twice with phosphate buffer saline, and lysed in extraction buffer containing 50 mM Tris–HCl, pH 7.4, 1.15% KCl, 5 mM glucose, 1% Triton X-100, 0.5 mM EDTA, 1 mM PMSF and 2 mM DTT. Protein concentra-

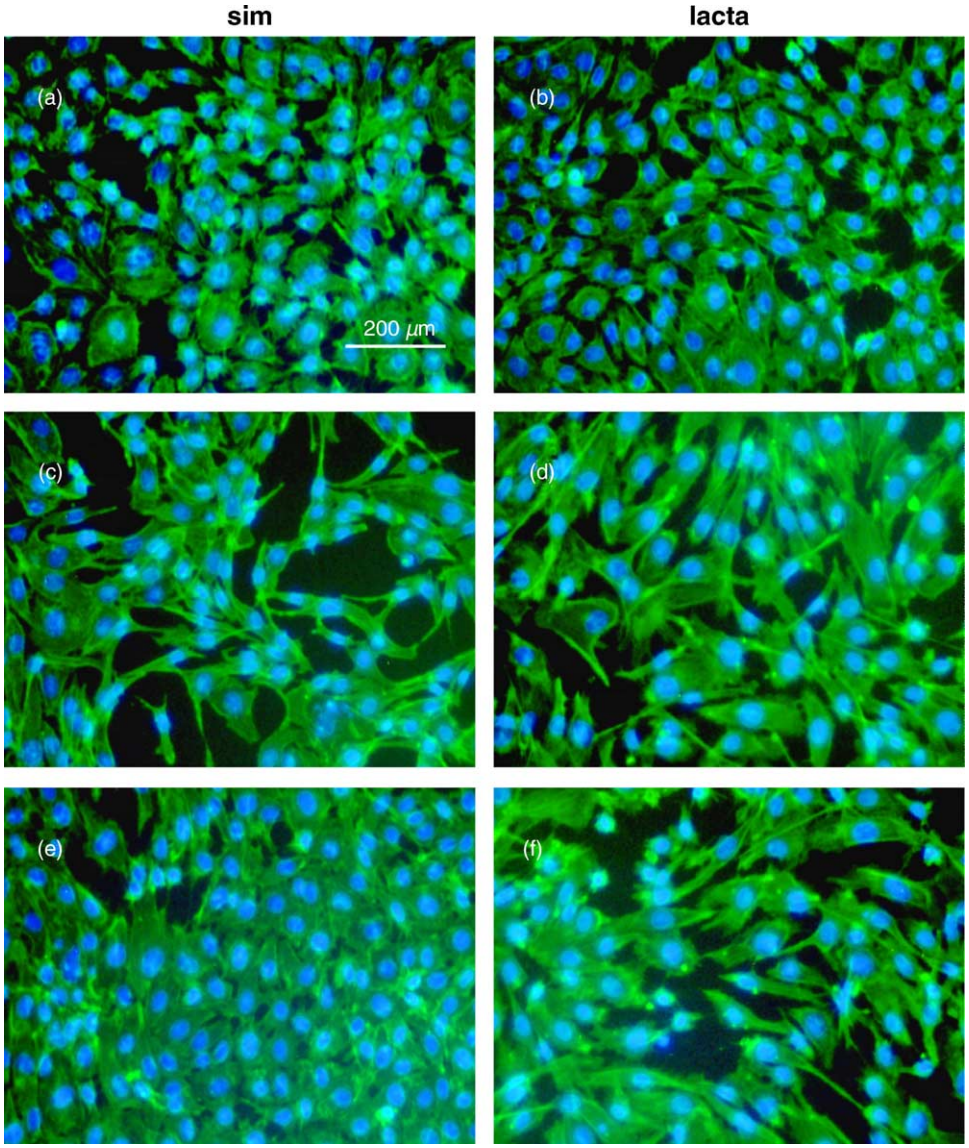

Fig. 1. The effects of 5 μM simvastatin and 10 μM lactacystin on the morphology of CPEA cells. (a) Ethanol-treated and (b) DMSO-treated CPAEs have a typical polygonal shape. (c) Treatment by simvastatin (5 μM) as well as (d) lactacystin (10 μM) induced elongation. Cotreatment with mevalonate (0.4 mM) abrogated the simvastatin-induced changes (e), whereas lactacystin-induced changes were not prevented by mevalonate (f).

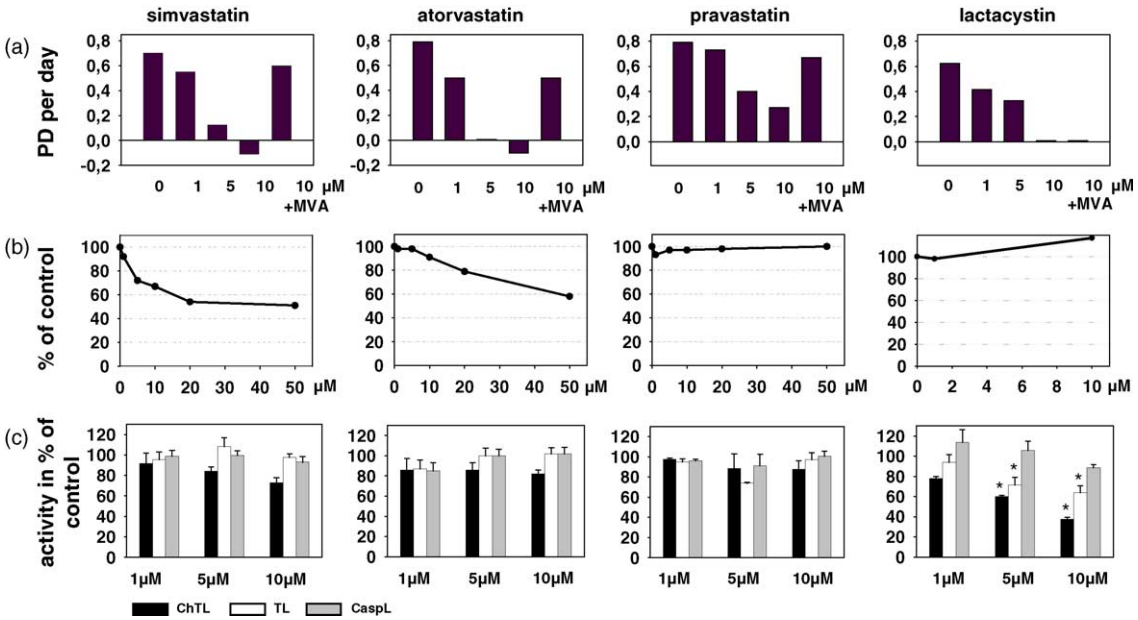

Fig. 2. Effect of simvastatin, atorvastatin, pravastatin and lactacystin on proliferation, viability and proteasomal activity of CPAE. (a) Rates of net proliferation of CPAE cells treated with increasing concentrations of simvastatin, atorvastatin, pravastatin and lactacystin. Cells were treated for 24 h (PD: population doublings per day). (b) Results of the XTT viability assay of CPAE cells treated with increasing concentrations of atorvastatin, simvastatin, pravastatin and lactacystin for 24 h. (c) Proteasomal activity in CPAE cells treated with increasing concentrations of simvastatin, atorvastatin, pravastatin and lactacystin for 24 h. Cells were lysed, followed by measurement of ChTL, TL and CaspL activities in the cell extract. Activities are expressed as percentage of solvent-treated control. $^{*}P < 0.05$ compared with solvent alone (mean ± S.E.M.).

tion was estimated by the BCA protein assay (Pierce). Total protein (60 μg per lane) was subjected to SDS-polyacrylamide gel electrophoresis. The proteins were then transferred to PVDF membranes. After blocking overnight, the membranes were probed with ubiquitin-specific antibodies. Antigens were detected with the enhanced-chemoluminescence system (Amersham-Pharmacia).

2.7. Statistics

Data variability about the mean was expressed as the standard error of the mean (S.E.M., standard deviation divided by the square root of the sample size). Significance was calculated using Student's *t*-test for comparison of control versus treated groups. We used SPSS 9.0 software for all statistical calculations.

3. Results

Fig. 1 shows the effects of a single application of 5 μM simvastatin pro-drug and of 10 μM lactacystin on the morphology of log-phase CPEA-cells. Photomicrographs were made after 24 h of treatment. CPAEs grown as a monolayer of adherent cells have a polygonal shape, which was not altered by vehicle treatment (Fig. 1a and b). Addition of simvastatin induced a change to an elongated phenotype (Fig. 1c). This effect was prevented by coincubation with 0.4 mM mevalonic acid (Fig. 1e). The same morphological changes were obtained by treatment with 10 μM of the selective proteasome inhibitor lactacystin. However, mevalonic acid failed to abrogate the changes induced by lactacystin in CPAE-cells (Fig. 1d and f). Treatment with 5 μM atorvastatin caused morphological changes comparable to those from simvastatin, whereas the effect of 5 μM pravastatin was less pronounced (not shown). The morphological changes induced by atorvastatin and pravastatin were prevented by mevalonate.

The effect of simvastatin, atorvastatin and pravastatin on the viability of CPAE cells was quantified by an XTT assay. Results showed that 24 h treatment of CPAE cells with simvastatin and atorvastatin decreased the viability in a dose-dependent manner, whereas pravastatin under the same experimental conditions did not affected viability (Fig. 2b). At concentrations >10 μM of simvastatin, viability was reduced by about 50%, whereas vehicle-treated CPAEs did not demonstrate a change in viability during the experiment.

Proliferation of CPAEs was dose dependently inhibited by all three statin drugs. Cotreatment with mevalonate (0.4 mM) reversed the inhibitory effect of 10 μM statin (Fig. 2a). Treatment of CPAEs with 10 μM lactacystin completely blocked proliferation, without signs of cytotoxicity, as measured in the XTT test (Fig. 2b). This growth arrest was not reversible by mevalonate (Fig. 2a).

To investigate the effect of statins on proteasomal activity in CPAEs, we treated cells with varying concentrations of simvastatin, atorvastatin, pravastatin, and lactacystin for 24 h, then lysed cells, and measured ChTL, TL and CaspL activities in the cell extract. Results disclosed

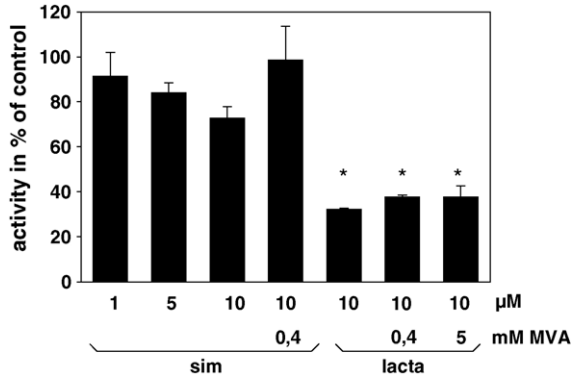

Fig. 3. Chymotrypsin-like activity in CPAE cells treated with various concentrations of simvastatin, mevalonic acid (MVA) and lactacystin. CPAE cells were treated with increasing concentrations of simvastatin, and with 10 μM lactacystin, and in combination with the indicated concentrations of MVA for 24 h. Cells were lysed and the chymotrypsin-like activity in the cell extract was measured. Activities are expressed as percentage of solvent-treated control. $^{*}P < 0.05$ compared with solvent alone (mean ± S.E.M.).

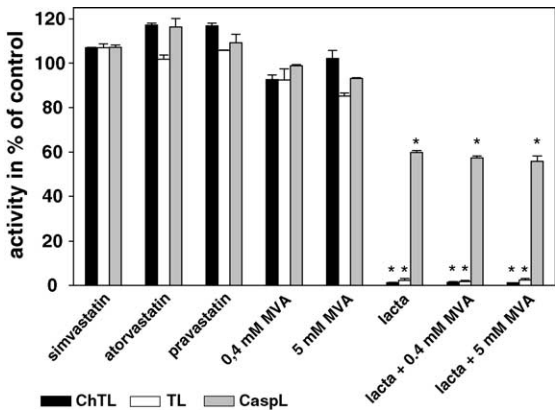

Fig. 5. Enzymatic activities of purified rabbit whole-blood 20S proteasomes incubated with either 100 μM simvastatin, 100 μM atorvastatin, 100 μM pravastatin or 10 μM lactacystin (lacta), 0.4 mM mevalonic acid (MVA) and 5 mM MVA or a combination of 10 μM lactacystin with either 0.4 or 5 mM MVA. Activities are expressed as percentage of solvent-treated control. $^{*}P < 0.05$ compared with solvent alone (mean ± S.E.M.).

that treatment of cells with atorvastatin and pravastatin did not inhibit any of the proteasomal activities (Fig. 2c). Simvastatin treatment led to a decrease of ChTL, whereas TL and CaspL remained unaffected (Fig. 2c). Lactacystin inhibited the chymotrypsin-like activity by approximately 60% of control at a concentration of 10 μM. The slight decrease of ChTL in CPAE by 5–10 μM simvastatin was statistically not significant and was able to be abrogated by MVA. The obvious inhibition of ChTL by lactacystin, on the other hand, was not modulated by MVA in concentrations up to 5 mM (Fig. 3). As shown by Western blot analysis, only the treatment with 10 μM lactacystin and not the treatment with statins, produced an accumulation of ubiquinated proteins (Fig. 4).

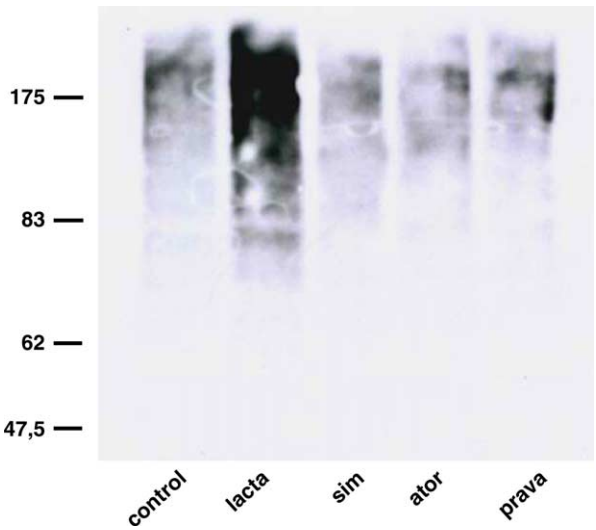

Fig. 4. Western blot analysis of accumulated multiubiquitinylated proteins of CPAEs treated with statins, and with proteasome inhibitor lactacystin. CPAEs were treated for 24 h with 10 μM atorvastatin (ator), simvastatin (sim), pravastatin (prava), lactacystin (lacta) and DMSO (control), washed, harvested after 24 h and analyzed for ubiquitin conjugates.

We investigated the influence of statins on proteasomal activities in another endothelial cell line, Ea.hy962, and in primary rat smooth muscle cells. In these cells as well, statins produced no change in any proteasomal activity (not shown).

To demonstrate the direct effect of simvastatin in its β-lactone closed ring form (pro-drug), atorvastatin and pravastatin on the proteasomal activity, we added a high concentration (100 μM) of each statin to purified human whole blood 20S proteasomes. The effects of simvastatin pro-drug, atorvastatin, pravastatin and lactacystin on the ChTL, TL and CaspL activities of purified 20S proteasomes are shown in Fig. 5. The selective proteasome inhibitor lactacystin, as expected, inhibited all three activities, whereas the statins under the same experimental conditions failed to do so. The ChTL and CaspL activities exhibited rather a slight increase.

One report has described mevalonate to be an activator of proteasomal activity when applied to human mamma carcinoma cells [9]. Our study revealed that MVA in concentrations up to 5 mM has no effect on proteasomal activity when added directly to purified 20S proteasomes, and was not able to abrogate the inhibitory action of lactacystin (Fig. 5).

4. Discussion

The question whether statins deploy their pleiotropic effects at least partially via modulation of the proteasomal activity has been addressed by several research groups with conflicting results. First evidence that the proteasome is inhibited by statins was provided by Rao et al. [9]. They reported that the closed ring form of lovastatin inhibits ChTL activity of the proteasome in the mamma carcinoma cell line MDA-MB157, resulting in cell cycle arrest. The

inhibitory effect of the lovastatin pro-drug was also demonstrated in crude extracts of these cells, indicating direct interaction of the drug with the proteasome complex. This study furthermore demonstrated an increase in proteasomal activity by mevalonate when applied to mamma carcinoma cells. However, Wojcik et al. [10] found that neither lovastatin pro-drug nor lovastatin treatment of colon carcinoma cells was able to induce the accumulation of polyubiquitinated proteins observed upon lactacystin application. Even though mevastatin inhibited proteasome activity in the extract of differentiated neuroblastoma cells in a dose-dependent manner, no change in proteasomal activity was detectable in mevastatin-treated neuroblastoma cells [11]. In osteoblasts treated with lovastatin an increase of ChTL was estimated, thereby refuting the hypothesis that lovastatin exerts its anabolic effect on bone by inhibiting the proteasome [12]. Other investigators could not observe any influence of statins on proteasomal activity [17,18].

In our study, we treated endothelial cells with statins and lactacystin and in fact observed similar effects: i.e., changed morphology to an elongated shape, attenuated proliferation and decrease of viability with higher concentrations. We show here, however, that neither statins in their closed ring form (simvastatin and atorvastatin) nor the open ring statin pravastatin influence the activity of purified 20S proteasomes. Likewise upon application to endothelial or primary smooth muscle cells, we detected no significant modulation of proteasomal activity measured in cell lysates. No accumulation of polyubiquitinated proteins occurred after statin treatment of endothelial cells. On the other hand, lactacystin, a specific irreversible inhibitor of proteasome activity, reduced proteasome activity 20S in proteasomes as well as in the intact cells, resulting in the accumulation of polyubiquitinated proteins in endothelial cells.

We conclude that neither closed nor open ring statins are modulators of the 20S proteasome, and that they are not able to induce changes of proteasomal activity when applied to mammalian endothelial and primary smooth muscle cells.

The slight decrease of ChTL in CPAE by 5–10 μM simvastatin (which was statistically not significant) was more likely a consequence of cytotoxicity or rather the induction of apoptosis [30] and was able to be abrogated by MVA. The obvious inhibition of ChTL by lactacystin, on the other hand, was not modulated by MVA. Moreover, in the other endothelial cell line, Ea.hy926, and in rat VSMC, simvastatin showed no effect of ChTL.

In our present study, mevalonic acid alone does not modulate proteasomal activity when added to 20S proteasomes or in cells. This is in contrast to results obtained on the extract of breast cancer cells in which mevalonic acid-stimulated proteasome activity in vitro [9]—but it concurs with other findings [10]. We demonstrated that the addition of mevalonic acid lactone immediately after statins completely prevents statin-induced morphological changes and a decrease in viability of CPAE cells. In contrast, mevalonic acid does not prevent lactacystin-induced changes of CPAE cells.

To summarize, the application of statins and the proteasome inhibitor lactacystin exhibited similar effects with respect to morphological changes and proliferation in endothelial cells. Mevalonic acid was able to reverse the statin-induced effects, but failed to abrogate lactacystin-induced effects. This indicates that statins and proteasome inhibitors at least partially affect the same pathways, but from independent initial points.

We conclude that the similar biological effects of the examined statins and proteasome inhibitors in vascular cells are not a result of a common mechanism of action on the proteasome.

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