Reversal of Thrombin-Induced Deactivation of CD39/ATPDase in Endothelial Cells by HMG-CoA Reductase Inhibition

Effects on Rho-GTPase and Adenosine Nucleotide Metabolism

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Abstract—Adenosine triphosphate and diphosphate that activate platelet, leukocyte, and endothelium functions are hydrolyzed by endothelial CD39/ATPDase. Because CD39/ATPDase is downregulated in endothelial cells by inflammation and this may be affected by HMG-CoA reductase inhibitors, we examined the role of cerivastatin and simvastatin in regulation of endothelial CD39/ATPDase expression, metabolism of ATP/ADP, and function in platelets. Thrombin-stimulated endothelial cells in vitro were treated with the statins, and hydrolysis of exogenous ADP and ATP was assessed by high-performance liquid chromatography and malachite green assay. Platelet aggregation studies were performed with endothelial cell supernatants as triggers. CD39/ATPDase surface expression by endothelial cells was determined immunologically by fluorescence-activated cell sorter, mRNA expression by RT-PCR, and thrombin-induced dissociation of Rho-GTPases by Western blotting. Treatment by simvastatin or cerivastatin restored impaired metabolism of exogenous ATP and ADP in thrombin-activated endothelial cells by preventing thrombin-induced downregulation of CD39/ATPDase. In platelet aggregation studies, ATP and ADP supernatants of thrombin-activated endothelial cells were less stimulatory in the presence of statins than in their absence. Data show that statins preserve CD39/ATPDase activity in thrombin-treated endothelial cells involving alterations by statins of Rho-GTPase function and CD39/ATPDase expression. Preservation of adenine nucleotide metabolism may directly contribute to the observed anti-thrombotic and anti-inflammatory actions of statins. (Arterioscler Thromb Vasc Biol. 2002;22:894-900.)

Key Words: ■ atherosclerosis ■ arterial thrombosis ■ thrombin ■ vascular biology ■ aggregation

Statins may have beneficial effects in atherogenesis by their anti-thrombotic and anti-inflammatory actions involving nonlipid mechanisms that modify endothelial function, inflammatory responses, and thrombus formation.1,2 Platelet activation and aggregation is considered important in atherogenesis. Platelets undergo activation at sites of injury and release ADP and ATP that further activate endothelial cells (ECs), platelets, and neutrophils via purinergic type-2 (P2) receptors.3,4 Opposite effects are exerted by AMP and adenosine via P1 receptors.5 ADP and ATP are present in extracellular fluid as a consequence of exocytosis of platelet dense bodies. CD39/ATPDase expressed by quiescent ECs hydrolyses extracellular ATP and ADP to AMP, which is further converted to adenosine by 5-nucleotidase.6,7 The preserved function of CD39/ATPDase is critical in the inhibition of platelet activation by keeping adenosine nucleotide levels low.8 Evidence was provided for the loss of CD39/ATPDase activity after EC activation such as vascular inflammatory states or xenograft rejection, where platelet deposition occurs.9–12 Changes in cholesterol homeostasis adversely influence vascular CD39/ATPDase activity and perturb other thromboregulatory proteins with implications for platelet sequestration within abnormal vessels.13 Statins act on platelets by modulating the thrombotic state in hypercholesteremic patients. Statins improve endothelial function and may ameliorate aberrant components of the coagulation cascade even in normocholesteremic statin-treated patients.14 Because in quiescent ECs, CD39/ATPDase acts to disperse platelet aggregates through degradation of ADP and ATP, which is reduced by inflammatory or coagulatory stimuli, we were interested in finding out whether statins might influence the adenosine nucleotide metabolic pathway in thrombin-stimulated human umbilical vein ECs...
(HUVECs). We report that statins preserve CD39/ATPDase activity in ECs in vitro.

Methods

Materials

Human thrombin, ADP, and gelatin were from Sigma. Cerivastatin (CVN) was from Bayer AG (Pharma Research) and simvastatin (SVN) from Merck (Research Laboratories). The high-performance liquid chromatography (HPLC)-grade solvents (methanol and water), NaOH, NaH2PO4, malachite green, ammoniumheptamolybdate tetrahydrate, and sulfuric acid were from Merck, Hanks’ balanced salt solution without phenol red was from Life Technologies Ltd. ATG, and Tween-20 were from Sigma. Hanks balanced salt solution without calcium and magnesium (HBS) and fetal calf serum (FCS) was from PAA Laboratories GmbH. Monoclonal antibody to cytosolic guanine nucleotide dissociation inhibitor (Rho-GDI) and anti-mouse IgG were purchased from Sigma. Phycoerythrin-conjugated anti-mouse IgG, human IgG, antihuman mouse IgG were purchased from Ancell Corp. ATP and Tween-20 were from Calbiochem-Novabiochem Corporation.

EC Growth Medium (EGCM) was from PromoCell, and fetal calf serum (FCS) was from PAA Laboratories GmbH. Monoclonal antibody to CD39 was from Ancell Corp. Phycoerythrin-conjugated anti-mouse IgG, human IgG, and anti-human mouse IgG were purchased from Sigma.

EC Culture

HUVECs from fresh placenta cords were isolated and grown to confluence at 37°C (humidified atmosphere). The growth medium was ECGM with 10% FCS. Culture flasks and 96-well plates were coated with 0.2% gelatin before seeding of cells. Cells were passaged by treatment with 500 μg/mL collagenase for 3 minutes. HUVECs of passages 1 and 2 were used.

Platelet Rich Plasma (PRP)

Human platelets were obtained from peripheral blood of healthy volunteers, anticoagulated with acid citrate, by centrifugation at 110g. Platelets were counted, and platelet-poor plasma was centrifuged at 1500g. Plastic or siliconized glassware was used for all platelet manipulations.

Treatment of HUVECs

HUVECs were passaged and plated at a density of 2.5×10⁵ cells/1000 μL medium into 12-well culture plates. When cells were grown to confluence, media were removed and cells washed with Hanks’ balanced salt solution without Ca²⁺ and Mg²⁺ and incubated with ECGM/0.2% FCS for 48 hours. HUVECs were treated with thrombin (0.1 mU/mL) and CVN or SVN (1 μmol/L to 100 μmol/L) for 45 minutes. Medium- or thrombin-treated cells served as controls. Then cells were washed twice and incubated with RPMI 1640 containing ADP (100 μmol/L) and ATP (100 μmol/L) for 20 minutes.

To analyze distinct metabolism of ADP and ATP, HUVECs were treated with either 100 μmol/L ADP or ATP. Supernatants were then harvested and subjected to either HPLC analysis or malachite green or platelet aggregation assays. Time course experiments were performed with SVN (1 μmol/L) and either ADP (100 μmol/L) or ATP (100 μmol/L). Supernatants in time course experiments were collected every 10 minutes for a total of 1 hour and analyzed by HPLC (ATP-treated) or malachite green assay (ATP-treated).

Measurement of Adenine Compounds in Platelet and HUVECs Supernatants by HPLC

HUVECs supernatants, 300-μL aliquots, were subjected to HPLC analysis. Separation of adenine nucleotides was performed on a Lichrosphere RP-18 column (200×4.6 mm; 5 μm; Agilent Technologies) connected to a ZORBAX SB-C18 precolumn (12.5×4.6 mm; 5 μm; Agilent Technologies). Signals were detected with a UV-visible detector (HP series 1100, Hewlett-Packard). The chromatographic conditions were as follows: isocratic elution at room temperature with 0.1 mol/L NaH2PO4 (pH 6.0 NaOH)/methanol (96%/4%); flow rate, 0.7 mL/min; pressure, ~122 bar; UV-visible detector wavelength, 254 nm; injection volume, 30 μL.

ATPase Activity

Inorganic phosphate and pyrophosphate generated by CD39/ATP-Dase in culture supernatants were measured by the malachite green colorimetric assay.17

Platelet Aggregation

Platelet aggregation was studied in PRP containing 250×10⁴ platelets/mL by Born’s method with a Lumi-Aggregometer (ChronoLog Corporation). PRP was preincubated at 37°C in an aggregometer cuvette. Platelet activation was started by addition of HUVEC supernatants. Addition of ADP served as positive control. Platelet aggregation was monitored by the increase of light transmission.

Western Blot Analysis of Rho-GTPases

HUVECs were incubated with thrombin (0.1 U/mL) and CVN or SVN (1 μmol/L to 100 μmol/L) for 20 minutes. Thrombin- or medium-treated cells served as controls. Cells were lysed in Nonident-P40 buffer (Roche). Proteins were separated on 7.5% SDS polyacrylamide gels and blotted onto polyvinylidene fluoride membranes. After blocking, the antibody was diluted to a final concentration of 0.05 μg/mL. Blots were incubated overnight at room temperature. Immunoreactivity was determined by using peroxidase-conjugated goat-anti-mouse IgG and the enhanced chemiluminescence reaction (Amersham). Intensity of the Western blot bands was quantified by using the Fluor-S Multimager System and the Quantity One Software (BioRad Laboratories).

Fluorescence-Activated Cell Sorter (FACS) Analysis of CD39

Confluent cells were treated with medium or thrombin or co-incubated with SVN. A total of 5×10⁵ HUVECs were washed in phosphate-buffered saline/0.5% bovine serum albumin and resuspended in 150 μg/mL human IgG for 20 minutes at 4°C. After pelleting, HUVECs were incubated with 10 μg/mL anti-CD39 or with the respective isotype-matched control IgG for 30 minutes at 4°C followed by a 1:40 dilution of anti-mouse R-phycocerythrin-conjugated IgG for 30 minutes. Then cells were analyzed by FACS and Cellquest software (Becton-Dickinson FACSscan).

Soluble CD39/ATPDase in EC Culture Supernatants

SANDWICH ELISA was performed according to standard protocols with supernatants of cells treated with medium, thrombin (0.1 μmol/L), SVN (1 μmol/L), and the combination of SVN (1 μmol/L) and thrombin (1 μmol/L) that were collected after 10, 20, 30, and 60 minutes (please see http://atvb.ahajournals.org). To investigate whether the soluble ATPDase is enzymatic active, ADP and ATP was added to the supernatants of thrombin- or thrombin- and statin-treated cells. HPLC measurement of adenine nucleotides in supernatants containing shedded CD39 was then performed.

Semi-quantitative Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from 8×10⁵ cells by phenol-chloroform-isoamylalcohol extraction (RNAcleanTM; Hybaid-AGS). RT-PCR was performed on 1 μg of RNA by using random hexamers reverse-transcriptase (Gibco BRL, Life Technologies). Ten microliters of the RT reaction mixture was then subjected to 35 cycles of PCR in a 50-μL reaction mixture containing 1.0 mol of sense and anti-sense primer pairs in a Perkin-Elmer thermocycler: 95°C for 30 seconds (denaturation), 53°C for 60 seconds (annealing), 72°C for 30 seconds (extension). Hot Start Taq polymerase was from Qiagen Inc. Primers (MWG Biotech) were designed to amplify a 428-bp coding sequence of CD39: sense, GCT CCA AGA ATA TCC TAG; anti-sense, CTT TTC ATC CTG AAC AAC. The PCR products were subjected to agarose gel analysis.
Effects of Statins on Thrombin-Inhibited Metabolism of ATP and ADP

Treatment of HUVECs with thrombin led to a decrease in free inorganic phosphate and pyrophosphate that was reversed in a concentration-dependent manner by treatment with either statin (Figure 1).

During incubation periods of up to 65 minutes, no measurable amounts of endogenous adenine nucleotides were released from HUVECs (data not shown). Under resting conditions (45 minutes of pretreatment with medium only), HUVEC monolayers metabolized exogenous ATP from 100 μmol/L to 30±12.4 μmol/L (n=7). Incubation of HUVECs for 45 minutes with thrombin, before addition of exogenous ATP, diminished this rate of ATP metabolism to 71±6.04 μmol/L (versus pretreatment with medium, n=7, P<0.01) (Figure 2). After addition of 100 μmol/L of ADP, ADP was hydrolyzed to 52.3±5.6 μmol/L, whereas pretreatment with thrombin diminished this rate to 86.9±4.3 μmol/L (versus pretreatment with medium, n=7, P<0.01) (Figure 2).

Because the simultaneous presence of exogenous ADP may affect the rate of ATP hydrolysis by CD39/ATPDase, enzymatic activities were also tested after addition of a combination of ATP and ADP. As compared with the untreated control, there were increased ATP and ADP levels in HUVEC supernatants by ≈240% and ≈180%, respectively, and a concentration-dependent increase of adenosine and a decrease of ATP and ADP levels when HUVECs were treated with SVN (please see http://atvb.ahajournals.org).

Similar patterns of enzymatic activities were seen with their combined addition. To test whether this effect is independent of cholesterol synthesis-inhibiting properties of the statins, we incubated HUVECs concomitantly with thrombin, statins, and mevalonic acid lactone (MVA). Statin-induced effects on thrombin-activated HUVECs were reversed by mevalonic acid. In this particular set of experiments (n=3), rates of ATP recovered in HUVEC monolayer supernatants after activation with 0.1 U/mL thrombin were 136±26.0% compared with untreated cells (P<0.05), which was reduced by the presence of 100 μmol/L SVN to 11±1.6% (P<0.05 versus thrombin); this reduction was affected by simultaneous presence of 500 μmol/L MVA resulting in a recovery rate of 101±24.9% (P<0.05 versus SVN). Corresponding numbers were 165±30.9%, 23±1.1%, and 155±7.1% for ADP; 133±11.6%, 105±13.3%, and 101±22.1% for AMP; and 173±26.1%, 446±97.0%, and 153±23.8% for adenosine, respectively. These effects of
MVA on adenine nucleotide metabolism was correspondingly confirmed in the malachite green assay of supernatants (Figure 1).

**Time Course Experiments and Kinetics of CD39/ATPDase Activity**

ADP, 100 μmol/L, was added to ECs, and supernatants were taken every 10 minutes for a total of 60 minutes. Already after 5 minutes of incubation, a significant increase of phosphate production was seen in supernatants of medium-, SVN-, or SVN- and thrombin-treated cells, whereas in thrombin-treated ECs, no such effect was observed (data not shown). HPLC measurement of ADP hydrolysis to AMP and adenosine revealed corresponding results (Figure 3). Enzymatic activities were calculated from rates of ADP degradation and phosphate generation (Figure I and Table I, please see http://atvb.ahajournals.org). Data demonstrate that SVN does not affect V_max of CD39/ATPDase, whereas thrombin reduced V_max significantly, which was partly reversed by addition of SVN.

**Figure 3.** Kinetics of ADP metabolism by ECs exposed to thrombin and statin. HUVEC monolayers were incubated with either medium, thrombin (0.1 mU/mL), SVN (1 μmol/L) or thrombin (0.1 mU/mL) and SVN (1 μmol/L); after 45 minutes cells were washed and ADP (100 μmol/L) added; supernatants were harvested every 10 minutes for 60 minutes and subjected to HPLC analysis (ADP-supplemented medium under cell culture conditions but without cells also measured). Recovery rates for ADP, AMP, and adenosine shown. Statistical analysis: Mann-Whitney U-test after Kruskal Wallis (P<0.01); *P<0.05, **P<0.01; (n=7).

**Shedding of CD39 From the ECs Induced by Thrombin**

Soluble CD39 was detected in supernatants of thrombin-treated ECs (Figure II, please see http://atvb.ahajournals.org).

**Statin-Dependent Effects of HUVEC Supernatants on Platelet Aggregation**

Aliquots of 100 μL of statin- and thrombin-treated supernatants supplemented with ADP and ATP were added to 1 mL of PRP and aggregation was measured. Supernatants of thrombin-treated HUVECs led to strong platelet aggregation (41.25 ± 1.25%), whereas statin-treated cell supernatants diminished this response in a concentration-dependent manner (Figure 4). As a positive control, platelet aggregation was triggered with ADP (100 μmol/L), where 75 ± 2.5% of aggregation was observed. When exogenous ADP and ATP were replaced with ADP-S, the effects of the supernatants on platelet aggregation were unaltered (Figure 4).

**Effect of Statins on Rho-GTPases**

Western blot analysis with a monoclonal antibody directed against free (unbound) Rho-GDI was performed. Quantification of monoclonal antibody binding to free Rho-GDI showed an increase in thrombin-treated (0.01 and 0.1 mU/mL) HUVECs (which means a decrease in membrane bound Rho-GTPases) and a concentration-dependent decrease (increase of Rho-GDI–bound) of activated Rho-GTPase in statin-treated cells (Figure 5).

**Effect of Statins on CD39 Expression**

Thrombin-treated (0.1 mU/mL) cells exhibited a decrease in anti-CD39 fluorescence activity compared with untreated quiescent cells (Figure 6A); treatment of thrombin-activated HUVECs with SVN restored anti-CD39 fluorescence activity (Figure 6B). After a 45-minute incubation period of HUVECs with thrombin (0.1 μmol/L), CD39 mRNA expression was reduced, but cotreatment with SVN (1 μmol/L) restored CD39 mRNA transcription (Figure 6C).
CD39/ATPase is the dominant vascular nucleoside triphosphate diphosphohydrolase that exerts major effects on platelet reactivity by regulating hydrolysis of extracellular adenine nucleotides.\textsuperscript{19,20} Purinergic signaling influences hemostasis and inflammatory responses. Therefore, hydrolysis of extracellular ATP and ADP by the CD39/ATPase would inhibit inflammatory and thrombotic processes.\textsuperscript{8} Statins may directly affect cells known to play key roles in the pathogenesis of atherothrombosis,\textsuperscript{21,22} including ECs and preservation of their anti-coagulatory properties.\textsuperscript{23–25} Because endothelial CD39/ATPase plays an important role in preventing vascular clot formation, we investigated possible roles of two lipophilic statins, CVN and SVN in endothelium-platelet interactions.

We confirmed that inflammatory stimulation of ECs leads to decreased ATP/ADP hydrolysis via downregulation of CD39/ATPDase using thrombin, which was previously not tested in this regard. This reduced adenine nucleotide metabolism led to an increase of platelet aggregation when PRP was supplemented with supernatants from thrombin-activated ECs. In contrast, with supernatants of cells incubated with ADP-\(H_9\) that is not hydrolyzed by CD39/ATPdase, platelet aggregation was not regulated. Effects of thrombin and adenine nucleotide metabolism were significantly diminished when HUVECs were cotreated with SVN or CVN; adenosine levels showed a concentration-dependent increase, whereas adenosine nucleotide levels were decreased. An increase of phosphate and pyrophosphate was correspondingly demonstrated. Platelet reactivity most likely was decreased as a result of decreasing amounts of ADP and the increase of the adenosine level. Direct effects of statins or thrombin on platelets are unlikely because these agents were removed from HUVEC cultures by aspiration of supernatants followed by washing of cells, before adenine nucleotides were added.

ATPDases of quiescent ECs act synergistically to disperse platelet aggregates through degradation of ADP and ATP,\textsuperscript{11} and cells activated with a pro-inflammatory stimulus or by exposure to polyunsaturated fatty acids, lose this function.\textsuperscript{13,26} Our data suggest that thrombin may affect anti-thrombotic properties of ECs by deactivation of endothelial CD39/ATPase, which can be prevented by statins. Via this mechanism, statins may reduce extracellular free ADP and ATP and, as a consequence, increase extracellular adenosine. Significant differences between thrombin- and thrombin- plus statin-treated cells were observed after time periods as short as 20 minutes, suggesting that this adenosine metabolism might underlie rapid alterations. Adenosine has previously been shown to inhibit a variety of cellular functions, including platelet aggregation, expression of tissue factor or adhesion molecules, and cytokine release by activated ECs.\textsuperscript{11} Our observation of decreased ATP and ADP concomitant with an increase of adenosine correlates with the simultaneous measurement of reduced in vitro aggregation in response to the culture supernatants.

As thrombin is a potent agonist for a number of biological responses that may mediate inflammatory and reparative responses to vascular injury,\textsuperscript{27} patients with increased thrombin generation are considered to be at enhanced cardiovascular risk.\textsuperscript{28} It was shown that thrombin levels in hypercholesteremic patients are enhanced.\textsuperscript{29} Protease-activating receptor (PAR) signaling pathways that mediate cellular effects of thrombin involve the activation of Rho-GTPases; PAR-1 receptors are not only found on platelets but also on

![Figure 5. Dissociation of Rho-GTPases in thrombin-activated ECs by CVN, Western blot analysis. HUVEC monolayers were incubated with thrombin (0.1 mU/mL) in absence or presence of SVN (1 pmol/L to 100 \(\mu\)mol/L) for 20 minutes; equal concentrations of protein were separated on SDS polyacrylamide gels; intensity data is expressed in counts per mm\(^2\) (CNT/mm\(^2\)) obtained in Fluor-S Multimager System (n=3). Statistical analysis: Mann-Whitney U-test after Kruskal Wallis (\(P<0.01\)); \(t^P<0.05\).](image)

![Figure 6. Effects of SVN on expression of CD39/ATPase by thrombin-activated ECs. Immunoreactivity quantified by FACS and mRNA expression quantified by RT-PCR. A, Quiescent HUVEC monolayers were treated by medium or thrombin (0.1 mU/mL). B, Effects of SVN (100 \(\mu\)mol/L) on thrombin-dependent CD39 cell surface expression. C, Regulation of mRNA expression through thrombin and cotreatment with SVN (1 \(\mu\)mol/L) as analyzed by RT-PCR. Primers were designed to amplify a 428-bp coding sequence of CD39.](image)
There is evidence that statins may affect cells via interference with signaling pathway proteins such as nuclear lamin B, ras proto-oncogen, Rho-related proteins, and the γ subunit of heterodimeric GTP-binding proteins. ECs are potentially exposed to a variety of extracellular proteases, particularly during vascular injury or inflammation. Thrombin is among these. Thrombin mediates increased endothelial retraction resulting in increased endothelial permeability to macromolecules. Activation of PAR by thrombin dissociates Rho-GTPases by detection of free cytosolic Rho-GDI, the effects of statins on adenosine metabolism as described here may be results of the inhibition of thrombin signaling. Preservation of CD39/ATPase might be the effect of inhibition of thrombin signaling by inhibition of HMG-CoA reductase–dependent pathways.

Exogenous MVA, a product of the mevalonate pathway, results in a redistribution of these GTPases to the membrane. Correspondingly, we observed MVA-induced reversal of statin-induced modulation of Rho-GTPase–dependent effects of thrombin. A decrease of CD39 surface expression was observed. This may be due to a decrease of CD39 synthesis as detected by reduced mRNA in RT-PCR experiments and an increase of shedding of CD39 in thrombin-treated cells. These observations were made after exposure of cells to thrombin and were to varying degrees affected by statins. Shedding of CD39/ATPase was already previously reported, which may remain enzymatically active. It might also be that thrombin cleaves CD39/ATPase at the apyrase-conserved region-1, resulting in substantive loss of biochemical activity. Quantitative contributions of thrombin-affected shedding and mRNA synthesis of CD39/ATPase in ECs to altered adenosine nucleotide metabolism awaits further study.

Adenosine protects tissue from hypoxic or ischemic damage and has been postulated to trigger preconditioning by increasing mitochondrial K-ATP channel activity; preconditioning through adenosine can protect tissue from ischemia/reperfusion damage when added during the reperfusion period. As we found an increase of free adenosine levels in statin-treated EC supernatants, it might be that via this mechanism statins also exert beneficial effects in patients who have ischemia/reperfusion injury. It is known that thrombin–via PAR receptors–activates the transcription factor NF-κB and, hence, is a prototypical pro-inflammatory mediator that inhibits endothelial CD39/ATPase, the regulation of which is NF-κB–dependent. Statins are able to prevent endothelial dysfunction and NF-κB activation in response to inflammatory stimuli by disrupting the Rho-GTPase–dependent signal transduction pathways.

In conclusion, our data suggest that treatment of cells with thrombin suppresses endothelial CD39/ATPase activity which can be completely preserved by the concomitant presence of CVN or SVN at doses that are achieved clinically. Adenosine nucleotides, which modulate platelet adhesion, recruitment to injured subendothelium, and formation of occlusive thrombi, are affected by statins in vitro. CD39/ATPase is the major enzyme that converts ADP and ATP to AMP and adenosine; inhibition of CD39/ATPase by thrombin suggests that its regulation during activation of coagulation may play a central role in the switch from an anti-thrombotic to a pro-thrombotic endothelial state. Functional antagonism of thrombin-triggered increase of ADP and ATP by statins represents a novel mechanism of action that may be of clinical relevance.

References


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