Antioxidant Effects of Statins via S-Nitrosylation and Activation of Thioredoxin in Endothelial Cells
A Novel Vasculoprotective Function of Statins

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Background—HMG-CoA reductase inhibitors (statins) are lipid-lowering drugs that also exert pleiotropic vasculoprotective effects via activation of the endothelial NO synthesis. NO induces S-nitrosylation of target proteins. S-Nitrosylation of the antioxidant enzyme thioredoxin was recently shown to enhance its activity, thereby reducing intracellular reactive oxygen species. Therefore, we investigated whether statins may exert an antioxidant activity in endothelial cells via S-nitrosylation of thioredoxin.

Methods and Results—Statins dose- and time-dependently increased the overall level of S-nitrosylated proteins in endothelial cells (atorvastatin 0.1 μmol/L, 206±30% increase; simvastatin 1 μmol/L, 214±19% increase; mevastatin 1 μmol/L, 191±10% increase). The increased S-nitrosylation was blocked by an NO-synthase inhibitor and mevalonate. Moreover, S-nitrosylation of thioredoxin was also significantly augmented after atorvastatin treatment. The atorvastatin-mediated increase in S-nitrosylation was associated with an enhanced enzymatic activity of thioredoxin (atorvastatin, 157±9% increase). This resulted in a significant reduction of intracellular reactive oxygen species within the endothelial cells. In contrast, in endothelial cells overexpressing a thioredoxin construct in which the S-nitrosylation acceptor amino acid cysteine 69 was replaced by serine [TRX(C69S)], atorvastatin did not activate the redox-regulatory activity of thioredoxin. Moreover, overexpression of the non-nitrosylatable thioredoxin TRX(C69S) abolished atorvastatin-mediated reduction of reactive oxygen species.

Conclusions—Here, we demonstrate a novel antioxidant mechanism by which statins reduce reactive oxygen species in endothelial cells. Statin-mediated S-nitrosylation of thioredoxin enhanced the enzymatic activity of thioredoxin, resulting in a significant reduction in intracellular reactive oxygen species. (Circulation. 2004;110:856-861.)

Key Words: antioxidants ■ nitric oxide ■ endothelium

HMG-CoA reductase inhibitors (statins) are effective lipid-lowering agents. Recent experimental studies suggested that the beneficial effects of statins in patients at risk for cardiovascular disease are not only a result of an improved lipid profile but also mediated by direct vasculoprotective actions.1 Nitric oxide (NO) produced within the endothelium is a pivotal mediator of vasculoprotective functions.2 Importantly, genetic ablation of the endothelial NO synthase (eNOS) abrogates the protective effects of statins on vascular function and ischemic tissue injury, suggesting an important role for eNOS to mediate the beneficial effects of statins.3-5 Indeed, statins were shown to upregulate eNOS posttranscriptionally in a Rho-GTPase-dependent manner.6 Moreover, statins activate the protein kinase Akt,7 which in turn phosphorylates and activates the eNOS.8,9 In addition to its enzymatic function and ischemic tissue injury, suggesting an important role for eNOS in coronary heart disease,8 the increased enzymatic activity of eNOS is associated with an enhanced redox-regulatory activity of thioredoxin (TRX).9,10 TRX is a redox-active protein consisting of 2 oxidoreductases, thioredoxin reductase and thioredoxin-1 (TRX). TRX is expressed ubiquitously in mammalian cells, and TRX deficiency results in a lethal phenotype,11 underscoring the fundamental importance of the enzyme. TRX exerts its enzymatic activity as an oxidoreductase via the cysteines 32 and 35. TRX can directly bind and metabolize ROS. Moreover, TRX interacts directly with other proteins, such as ribonucleotide reductase, protein disulfide isomerase, and several transcription factors (including p53, nuclear factor-κB, and activating protein-1 via Ref-1) by forming disulfide bridges.12-14 In addition to the 2

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cysteines at position 32 and 35 in the redox-regulatory domain, TRX contains 3 cysteines at position 62, 69, and 73, which are believed to be structurally important. NO induces the S-nitrosylation of TRX at cysteine 69 in endothelial cells, and this S-nitrosylation is required for the redox-regulatory activity of TRX and its ability to reduce intracellular ROS in endothelial cells. Therefore, we investigated the effects of statins on the content of S-nitrosylated molecules in endothelial cells and in particular on the S-nitrosylation of TRX. The results of the present study demonstrate that statins increase S-nitrosylation of TRX at cysteine 69. The increase in S-nitrosylation of TRX is accompanied by an increase in the redox-regulatory activity of TRX and by a decrease in intracellular ROS. Therefore, these data provide evidence for a direct antioxidative effect of statins via S-nitrosylation of TRX in endothelial cells.

**Methods**

**Cell Culture**

Human umbilical vein endothelial cells (endothelial cells) were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/mL), bovine brain extract (12 μg/mL), gentamicin (50 μg/mL), amphotericin B (50 ng/mL), epidermal growth factor (10 ng/mL), and 10% FCS from passages 2 to 4. After detachment with trypsin, cells were grown for at least 18 hours. Atorvastatin (AT) (10 ng/mL), and 10% FCS were added to the culture medium. The adherent cells were loaded with 2',7'-dichlorodihydrofluorescein diacetate (H2 DCF-DA, 20 μmol/L, Molecular Probes) or dihydroethidium (DHE, 5 μmol/L, Molecular Probes) for 30 minutes at 37°C in the dark. Cells were trypsinized for 2 minutes, and the reaction was stopped with PBS/10% FCS. Cells were pelleted by centrifugation and washed twice with PBS. The pellet was resuspended in 500 μL PBS. After addition of 140 mU of thioredoxin reductase, the samples were incubated in the dark at room temperature for 30 minutes, and 1 mol/L NaOH was added. The generated fluorescent triazole from the reaction of 2,3-diaminonaphthalene with the NO released from TRXwt or TRX mutants was measured by use of an excitation wavelength of 375 nm and an emission wavelength of 450 nm. As negative control, the Xpress antibody alone in lysis buffer was immunoprecipitated. The resulting background fluorescence intensity was subtracted from each experiment, and the equal expression of TRXwt and TRX mutants was confirmed by immunoblot using an anti-Xpress antibody (1:2000, Invitrogen).

**Detection of ROS Formation**

For measurement of the intracellular H2O2 and O2·− levels, the adherent cells were loaded with 2',7'-dichlorodihydrofluorescein diacetate (H2 DCF-DA, 20 μmol/L, Molecular Probes) or dihydroethidium (DHE, 5 μmol/L, Molecular Probes) for 30 minutes at 37°C in the dark. Cells were trypsinized for 2 minutes, and the reaction was stopped with PBS/10% FCS. Cells were pelleted by centrifugation and washed twice with PBS. Formation of ROS was detected by the signal obtained from the fluorescent reaction products DCF and ethidium by use of flow cytometry (FACSCalibur; Perkin Elmer; fluorescence 1 [DCF], 530/30 nm; fluorescence 2 [ethidine], 585/42 nm; CellQuest software). All experiments were performed with standardized instrumental settings (BD Biosciences).

**Immunoblot**

After stimulation with 0.1 μmol/L AT for 24 hours, endothelial cells were scraped off the plates and lysed in RIPA buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% Nonidet-P40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate). For detection of protein expression, membranes were incubated with antibodies against TRX (1:500, 2 hours, room temperature, Neomarkers) as described previously.

**Statistics**

Statistical analysis was performed with Student’s t test or ANOVA followed by modified least significant difference (Bonferroni) test (SPSS Software).

**Results**

**Effects of Statins on Intracellular S-NO Content in Endothelial Cells**

To address the question of whether statins increase the intracellular content of S-nitrosylated molecules (S-NO content), endothelial cells were incubated with different statins in a concentration-dependent manner for 24 hours. All 3 statins tested significantly increased the S-NO content in a dose-dependent manner (Figure 1A). Next, we determined the time course of increased S-NO content. Incubation with 0.1 μmol/L AT enhanced the S-NO content in a time-dependent manner, with maximum effect observed after 48 hours (Figure 1B). The AT-induced increase in S-NO content was completely inhibited by coinubcation with mevalonate (Figure 1C). Likewise, inhibition of the NO synthase with Nω-monomethyl-L-arginine abolished the AT-induced increase in S-NO content (Figure 1D), demonstrating that NOS activation is required for the increase in S-NO content by AT.
AT Increases S-Nitrosylation of TRX and Thereby TRX Enzyme Activity

TRX was shown to be S-nitrosylated at cysteine 69 in endothelial cells,20 which enhances the redox-regulatory and antiapoptotic functions of TRX. Therefore, we determined whether AT might influence S-nitrosylation of TRX. Indeed, S-nitrosylation of TRX was significantly increased by incubation with AT compared with control cells (Figure 2A). An effect of AT on TRX protein levels was excluded by Western blot analysis (Figure 2B). Taking into account that S-nitrosylation of TRX increased TRX enzyme activity in endothelial cells,20 we next examined the effect of AT on TRX enzyme activity. AT significantly increased TRX activity under basal conditions in endothelial cells, whereas a specific Rho kinase inhibitor, Y 27632, had no effect (Figure 3A). Moreover, AT also inhibited H2O2-induced reduction in TRX activity (Figure 3A). Similar results were found in cells overexpressing TRXwt (Figure 3B). In contrast, AT had no effect in endothelial cells overexpressing the non–S-nitrosoylatable TRX mutant (TRXC69S) (Figure 3B), indicating that AT-induced S-nitrosylation of TRX contributes importantly to AT-increased TRX enzyme activity.

AT Reduces Intracellular ROS by Activatory S-Nitrosylation of TRX Under Basal Conditions

We demonstrated previously that S-nitrosylation of TRX enhances its antioxidant capacity, which leads to reduced intracellular ROS.20 Indeed, incubation of endothelial cells

Figure 1. A, AT, simvastatin, and mevastatin increased S-NO content in endothelial cells. Endothelial cells were incubated with AT, simvastatin, and mevastatin at concentrations indicated for 24 hours. Intracellular S-NO content was measured as described in Methods (data are mean±SEM, n=6, *P<0.01 vs control). B, Endothelial cells were incubated with 0.1 μmol/L AT for indicated times, and S-NO content was measured as described in Methods (data are mean±SEM, n=3, *P<0.01 vs control). C, Endothelial cells were preincubated with 40 μmol/L mevalonate followed by 0.1 μmol/L AT for 24 hours. S-NO content was measured as described in Methods (data are mean±SEM, n=4, *P<0.01 vs AT). D, AT-induced increase in S-NO content is dependent on NOS activation. Endothelial cells were incubated with L-NMMA for 24 hours before treatment with 0.1 μmol/L AT for 24 hours. S-NO content was measured as described in Methods (data are mean±SEM, n=4, *P<0.01 vs AT).

Figure 2. A, AT induced S-nitrosylation of endogenous TRX. After immunoprecipitation with an antibody against thioredoxin, S-nitrosylation of endogenous thioredoxin was detected by use of diaminonaphthalene-assay (data are mean±SEM, n=6, *P<0.05 vs control). B, AT has no effect on TRX protein expression. Endothelial cells were incubated with 0.1 μmol/L AT for 24 hours. Immunoblotting with an anti-TRX antibody was performed. Membranes were reprobed with anti-tubulin antibody. A representative immunoblot is shown (top). TRX/tubulin ratio was quantified by scanning densitometry using Scion Image program (bottom; n=4).
with AT reduced intracellular ROS (Figure 4, A and B). To distinguish between superoxide anions and H$_2$O$_2$, we used 2 fluorescent dyes, H$_2$DCF-DA and DHE. H$_2$DCF-DA is described to detect primarily, but not exclusively, H$_2$O$_2$.27 As demonstrated in Figure 4, A and B, AT reduced intracellular ROS to a similar extent, suggesting that O$_2^-$/H$_2$O$_2$ and H$_2$O$_2$ are produced to a similar extent. Moreover, preincubation with AT also reduced H$_2$O$_2$-induced ROS levels below basal levels (Figure 4C). To document that AT-induced S-nitrosylation of TRX was required for the ability of AT to decrease intracellular ROS levels within endothelial cells, we transfected endothelial cells with empty vector, TRXwt, or TRX(C69S). As shown in Figure 4B, overexpression of TRXwt significantly reduced intracellular ROS levels, whereas overexpression of TRX(C69S) showed ROS levels comparable to those in vector-transfected cells. Incubation with AT significantly reduced ROS in vector-transfected and in TRXwt-transfected cells (Figure 4D). However, AT did not influence intracellular ROS levels in endothelial cells overexpressing TRX(C69S) (Figure 4D), demonstrating that AT-induced reduction of intracellular ROS is dependent on S-nitrosylation of TRX at cysteine 69 in endothelial cells.

**Discussion**

It is generally accepted that statins exert beneficial effects not only by improving the lipid profile but also because of pleiotropic effects.1 Recent studies demonstrated that statins have anti-inflammatory and antioxidant effects.28–30 Because increased ROS contribute to vascular inflammation and may injure endothelial cells, we investigated the effect of statins on endothelial ROS levels under basal conditions. The present study demonstrates that statins increase the overall content of S-nitrosylated proteins and the S-nitrosylation of the redox regulator TRX, thereby reducing the intracellular ROS levels within endothelial cells.

Recent studies demonstrated that statins reduce the generation of intracellular ROS in smooth muscle cells and in cardiac myocytes after stimulation of the NADPH oxidase with growth factors or angiotensin II.29–31 Moreover, cerivastatin was shown to reduce basal NADPH oxidase activity in human umbilical vein endothelial cells.32 Mechanistically, the reduction of ROS generation by statins was attributed to an
inhibition of the rac1 GTPase activation and translocation from the cytosolic compartment to the cell membrane, which is a prerequisite of NADPH oxidase activation.\textsuperscript{29,30,33} Associated with the Rac-GTPase inhibition is a reduction of the expression of NADPH oxidase isoforms by statins.\textsuperscript{29,30,33} All of these studies focused on induced ROS formation by different stimuli and thereby investigated the effect of statins on ROS-generating enzymes. The present data investigating intracellular \( \ce{O_2^-} \) and \( \ce{H_2O_2} \) levels under basal conditions in endothelial cells provide novel insights into the regulation of the antioxidant defense by statins. In contrast to catalase, which was shown to be transcriptionally increased after statin treatment in some but not all of the studies,\textsuperscript{30,32} TRX was not regulated on a transcriptional level. Instead, statins activated TRX via posttranscriptional activation of the enzymatic activity, which resulted in reduction of intracellular ROS levels. The reduction of intracellular ROS by AT was abolished in endothelial cells overexpressing a thioredoxin construct, in which cysteine 69 was mutated to non-S-nitrosylatable serine. These results demonstrate that AT-mediated reduction of ROS depends on S-nitrosylation of thioredoxin. Thus, S-nitrosylation of TRX may provide another mechanism in addition to the inhibition of Rac1 GTPase\textsuperscript{29,30,33} underlying the antioxidant effects of statins. Interestingly, all effects of statins on the redox system, which include the inactivation of the NADPH oxidase and the activation of thioredoxin, are reversed by mevalonate. Therefore, all these antioxidant actions of statins are dependent on HMG-CoA reductase inhibition.

The role of eNOS in cholesterol-independent vascular and ischemic tissue protection by statins was delineated previously in in vivo models using genetic or pharmacological approaches. Not only did pretreatment with statins increase eNOS activity, but protection by statins was also completely abolished in eNOS-knockout mice or after inhibition of eNOS with \( \ce{N^\omega,N^\omega-\text{nitro-L-arginine-methyl ester}} \).\textsuperscript{3,5} Of note, genetic ablation of eNOS results in a reduction of S-nitrosylated molecules.\textsuperscript{20} Inhibition of the NO release thus may result in a reduced antioxidant defense capacity. Therefore, NO not only interacts directly with ROS to reduce intracellular ROS via the formation of peroxynitrite but also indirectly targets intracellular ROS by activation of ROS-metabolizing enzymes, such as thioredoxin. Given that ROS are intimately involved in activation of the prototypic inflammatory transcription factor nuclear factor-\( \kappa \)-B,\textsuperscript{34} it is reasonable to postulate that statin-mediated S-nitrosylation of TRX also contributes to the well-established anti-inflammatory effects of statins by maintaining the redox balance within endothelial cells.

Increased oxidative stress leads to severe cell damage and induction of apoptosis. Several in vitro and in vivo studies demonstrated that TRX can potently reduce oxidative stress-induced cellular damage and apoptosis.\textsuperscript{20,35,36} TRX not only inhibits reperfusion-induced arrhythmias in a rat heart model\textsuperscript{19,37,38} but is also protective against adriamycin-induced cardiotoxicity.\textsuperscript{39} Thus, activation of TRX by NO-dependent S-nitrosylation may provide an important protective mechanism, which may not be restricted to endothelial cells. One may speculate that statin-mediated activation of TRX may also contribute to the direct cardioprotective effects of statins in cardiac myocytes.\textsuperscript{20}

Taken together, the data of the present study demonstrate that statins S-nitrosylate TRX via activation of NO synthesis, resulting in an enhanced redox-regulatory activity of TRX and decreased intracellular ROS levels. Moreover, AT-induced S-nitrosylation of TRX is a novel mechanism by which statins interfere with the antioxidant potential of endothelial cells.

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