Cellular Antioxidant Effects of Atorvastatin
In Vitro and In Vivo

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Abstract—3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) may exert direct effects on vascular cells and beneficially influence endothelial dysfunction. Because reactive oxygen species (ROS) may lead to vascular damage and dysfunction, we investigated the effect of atorvastatin on ROS production and the underlying mechanisms in vitro and in vivo. Cultured rat aortic vascular smooth muscle cells were incubated with 10 μmol/L atorvastatin. Angiotensin II–induced and epidermal growth factor–induced ROS production were significantly reduced by atorvastatin (dichlorofluorescein fluorescence laser microscopy). Atorvastatin downregulated mRNA expression of the NAD(P)H oxidase subunit nox1, whereas p22phox mRNA expression was not significantly altered (reverse transcription–polymerase chain reaction, Northern analysis). Membrane translocation of rac1 GTPase, which is required for the activation of NAD(P)H oxidase, was inhibited by atorvastatin (Western blot). mRNA expression of superoxide dismutase isoforms and glutathione peroxidase was not modified by atorvastatin, whereas catalase expression was upregulated at mRNA and protein levels, resulting in an increased enzymatic activity. Effects of atorvastatin on ROS production and nox1, rac1, and catalase expression were inhibited by L-mevalonate but not by 25-hydroxycholesterol. In addition, spontaneously hypertensive rats were treated with atorvastatin for 30 days. ROS production in aortic segments was significantly reduced in statin-treated rats (lucigenin chemiluminescence). Treatment with atorvastatin reduced vascular mRNA expression of p22phox and nox1 and increased aortic catalase expression. mRNA expression of superoxide dismutases, glutathione peroxidase, and NAD(P)H oxidase subunits gp91phox, p40phox, p47phox, and p67phox remained unchanged. Translocation of rac1 from the cytosol to the cell membrane was also reduced in vivo. Thus, atorvastatin exerts cellular antioxidant effects in cultured rat vascular smooth muscle cells and in the vasculature of spontaneously hypertensive rats mediated by decreased expression of essential NAD(P)H oxidase subunits and by upregulation of catalase expression. These effects of atorvastatin may contribute to the vasoprotective effects of statins. (Arterioscler Thromb Vasc Biol. 2002;22:300-305.)

Key Words: statins ■ reactive oxygen species ■ NAD(P)H oxidase ■ vascular smooth muscle cells ■ spontaneously hypertensive rats

As demonstrated by multiple interventional trials,1–4 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) effectively reduce the cardiovascular events of patients with coronary heart disease and hypercholesterolemia. The antiatherogentic potential of these drugs is not exclusively dependent on their lipid-lowering properties, which led to the proposition that statins may exert pleiotropic effects on vascular and cardiac cells independent of cholesterol synthesis.5,6 It has been shown that statins may induce apoptosis, inhibit cell cycle progression, reduce the proliferation of vascular smooth muscle cells (VSMCs),7–9 decrease inflammatory processes,10–12 increase the production of NO in endothelial cells by the upregulation of endothelial cell NO synthase (ecNOS) expression,13 reduce the production of endothelin-1,14 and beneficially influence the coagulation processes.15 These effects are dependent on mevalonic acid (the product of the enzyme reaction inhibited by statins), which serves as precursor of numerous isoprenoid metabolites.16

The increased release and production of reactive oxygen species (ROS) is thought to be one of the key events in the pathogenesis of endothelial dysfunction and atherosclerosis.17 ROS elicit direct cellular damage and mitogenicity, serve as intracellular second messengers, and scavenge vasoprotective NO.18 The predominant source of ROS production in the vessel wall is the NAD(P)H oxidase system.19,20 The latter is composed of various subunits, such as rac1, p22phox, gp91phox, nox1 (formerly termed mox1), p40phox, p47phox, and p67phox.21–24 Previous studies have suggested that the modulation of subunit expression is decisively important for
the overall activity of NAD(P)H oxidase.\textsuperscript{24–26} Besides ROS-generating enzymes, antioxidative defense systems are important for the oxidative stress that ultimately results. The superoxide dismutase (SOD) isoforms, glutathione peroxidase (GPX), and catalase (CAT) are enzymes residing within the vasculature that finally lead to the elimination of free radicals by the generation of water and oxygen.\textsuperscript{27,28} Because alterations of either the production or elimination of ROS are known to accelerate the progression of endothelial dysfunction and atherosclerosis, we hypothesized that statins could potentially interact with the gene products involved in these processes. Therefore, the influence of statins on ROS production and the expression of NAD(P)H oxidase subunits as well as radical-scavenging enzymes were investigated in cultured VSMCs and in spontaneously hypertensive rats (SHR).

**Methods**

The Methods section can be accessed online (please see http://atvb.ahajournals.org).

**Results**

**Effect of Atorvastatin on Production of ROS in VSMCs**

To evaluate the effect of atorvastatin on intracellular ROS production, VSMCs were preincubated for 12 hours with vehicle, 10 \(\mu\)mol/L atorvastatin, atorvastatin plus \(I\)-mevalonate (200 \(\mu\)mol/L), or atorvastatin plus 25-hydroxycholesterol (5 \(\mu\)g/mL), followed by a 3-hour coincubation with 1 \(\mu\)mol/L angiotensin II. ROS production was quantified by dichlorofluorescein fluorescence laser microscopy. Data analysis of 4 separate experiments is illustrated in Figure 1A. Stimulation with angiotensin II led to a marked increase in ROS production (246\% of control, \(P<0.05\) versus control). Preincubation with atorvastatin for 12 hours significantly reduced angiotensin II–induced ROS production to 111\% of control (\(P<0.05\) versus angiotensin II). \(I\)-Mevalonate completely reversed the effect of atorvastatin on angiotensin II–induced free radical production (246\% of control, \(P<0.05\) versus control), whereas 25-hydroxycholesterol had no effect (114\% of control, \(P=0.05\) versus angiotensin II). Incubation with atorvastatin, mevalonate, or hydroxycholesterol alone had no effect on basal ROS production (Figure 1C). Angiotensin II–mediated ROS production was completely prevented by coincubation with diphenylene iodonium (DPI, 10 \(\mu\)mol/L), an inhibitor of flavoprotein-containing oxidoreductases such as NAD(P)H oxidase (Figure 1C). In addition, VSMCs were preincubated for 12 hours with vehicle, 10 \(\mu\)mol/L atorvastatin, atorvastatin plus \(I\)-mevalonate (200 \(\mu\)mol/L), or atorvastatin plus 25-hydroxycholesterol (5 \(\mu\)g/mL), followed by a 3-hour coincubation with 20 ng/mL epidermal growth factor (EGF). The EGF–enhanced ROS production (236\% of control, \(P<0.05\) versus control) was also inhibited by preincubation with atorvastatin (95\% of control, \(P<0.05\) versus EGF), as shown in Figure 1B. Again, this was inhibited by \(I\)-mevalonate (233\% of control, \(P<0.05\) versus control), whereas hydroxycholesterol had no effect (101\% of control, \(P<0.05\) versus EGF).

**Effect of Atorvastatin on NAD(P)H Oxidase Subunit Expression in VSMCs**

NAD(P)H oxidase is a multicomponent enzyme complex that is thought to be a major source of ROS in the vessel wall and can be activated through angiotensin II type 1 receptor activation by angiotensin II. To examine whether statin treatment leads to an altered expression of essential subunits of the NAD(P)H oxidase in VSMCs, the cells were treated for 0 to 24 hours with 10 \(\mu\)mol/L atorvastatin or vehicle, and total cellular RNA was extracted at the indicated time points. p22phox mRNA levels were assessed by Northern analysis. Figure 2A shows a representative autoradiograph, indicating that compared with the corresponding GAPDH mRNA levels, p22phox mRNA expression was not significantly altered by atorvastatin. In addition, bovine aortic endothelial cells (BAECs) were treated for 0 to 24 hours with 10 \(\mu\)mol/L atorvastatin or vehicle; subsequently, Northern blot experiments were performed to assess p22phox mRNA expression in this cell type. A representative autoradiograph is shown in Figure 2B. The densitometric analysis of 3 separate experiments revealed that p22phox mRNA expression was slightly upregulated to 114\% of control after 8 hours but was finally decreased to 72\% of control after 24-hour incubation with atorvastatin (\(P<0.05\) versus control).

Nox1 mRNA expression in VSMCs after treatment with 10 \(\mu\)mol/L atorvastatin for 0 to 24 hours was measured by semiquantitative reverse transcription (RT)–polymerase chain reaction (PCR). Figure 2C shows a representative agarose gel of the amplified nox1 and GAPDH DNA frag-
VSMCs were treated for 8 hours with con, ator (10 μmol/L), or vehicle (con), and total cellular RNA was extracted at the indicated time points. p22phox mRNA levels were assessed by Northern analysis (data not shown). In addition, VSMCs were incubated for 0 to 24 hours with 10 μmol/L atorvastatin or vehicle, total cellular RNA was extracted at the indicated time points, and Northern analysis was performed. As demonstrated in representative Northern blots in Figure 3A, statin treatment did not significantly alter the mRNA expression of cat, meva, and chol (5 μg/mL) or with atorvastatin plus mevalonate, hydroxycholesterol, or GGPP. The effect of atorvastatin on rac1 membrane protein expression was inhibited by mevalonate and by GGPP but not by hydroxycholesterol, as shown in a representative immunoblot in Figure 2F. Finally, the effect of angiotensin II (1 μmol/L, 3 hours) in the presence or absence of atorvastatin (10 μmol/L, 15 hours) on rac1 protein expression in the membrane fraction of VSMCs was investigated. Treatment with angiotensin II enhanced rac1 expression, whereas coinulation with atorvastatin not only reversed this effect but further decreased rac1 expression below control levels, as demonstrated in Figure 2F.  

Effect of Atorvastatin on Expression and Activity of Antioxidative Enzymes in VSMCs

As a counterpart to ROS-generating enzymes, several antioxidative enzymes are involved to preserve the balance of ROS availability within vascular cells. Decreased production of free radicals in VSMCs after statin treatment could also be mediated by increased expression of the radical-scavenging enzymes manganese SOD (mSOD), extracellular SOD (ecSOD), copper-zinc SOD (czSOD), GPX, or CAT. Therefore, VSMCs were incubated for 0 to 24 hours with 10 μmol/L atorvastatin or vehicle, total cellular RNA was extracted at the indicated time points, and Northern analysis was performed. As demonstrated in representative Northern blots in Figure 3A, statin treatment did not significantly alter the mRNA expression of mSOD, ecSOD, GPX, czSOD, or GAPDH (representative of 3 separate experiments). In contrast, mRNA expression of CAT was significantly upregulated by atorvastatin, beginning after 4 hours of incubation (180±14% of control) and reaching a maximum of 227±6% of control after 12 hours (densitometric analysis of 3 experiments, P<0.05 versus control). A representative Northern blot is shown in Figure 3B. In addition, VSMCs were treated for 8 hours with vehicle, atorvastatin (10 μmol/L), L-mevalonate (200 μmol/L), or 25-hydroxycholesterol (5 μg/mL) or with atorvastatin plus mevalonate or hydroxycholesterol. As indicated in a representative autoradiograph in Figure 3C, the statin-mediated effect on CAT mRNA expression was reversed by mevalonate but not by hydroxycholesterol. The enhanced expression of CAT mRNA was translated to an increase of CAT protein expression (n=3) and CAT enzyme activity (4.1±1.0 [control] versus 6.7±0.6 [atorvastatin] U/mg protein, P<0.05 versus control; n=4) in VSMCs, as demonstrated in Figure 3D and 3E. Enzyme activity of the SODs and GPX was not altered by statin treatment (data not shown).
A

\[ \text{ECkSOD, cSOD, GPX, CAT, and the corresponding GAPDH mRNA expression, as assessed by Northern analysis in VSMCs incubated with either con or 10} \mu \text{mol/L ator.}\]

B

\[ \text{CAT enzyme activity was assessed by spectrophotometric quantification of H}_2\text{O}_2\text{ conversion in proteins of VSMCs incubated with either con or 10} \mu \text{mol/L ator.}\]

C

\[ \text{ROS was assessed by lucigenin chemiluminescence assays in isolated aortic segments of con- and statin-treated SHR.}\]

D

\[ \text{ROS production in vivo, we assessed the expression of NAD(P)H oxidase subunits and antioxidative enzymes in RNA of aortic homogenates of control and statin-treated SHR by means of semiquantitative RT-PCR (n=3 per group).}\]

E

\[ \text{Effect of atorvastatin on vascular ROS production in SHR. SHR received standard chow or standard chow supplemented with ator (50 mg/kg per day) for 30 days. Vascular production of ROS was assessed by lucigenin chemiluminescence assays (5 } \mu \text{mol/L lucigenin} \text{ in isolated aortic segments of con- and statin-treated SHR. RLU indicates relative light units. Values are mean±SEM (n=6). *P<0.05 vs con.}\]

Effect of Atorvastatin on Vascular Expression of NAD(P)H Oxidase Subunits and Antioxidative Enzymes in SHR

To examine the mechanisms underlying the decreased vascular ROS production in vivo, we assessed the expression of NAD(P)H oxidase subunits and antioxidative enzymes in RNA of aortic homogenates of control and statin-treated SHR by means of semiquantitative RT-PCR (n=3 per group). Densitometric analysis demonstrated that the expression of nox1 mRNA (53±14% of control, P<0.05 versus control) and p22phox mRNA (57±9% of control, P<0.05 versus control) was reduced in the aortas of SHR treated with the statin, whereas GAPDH expression remained unchanged. Other subunits of the NAD(P)H oxidase, namely, gp91phox, p40phox, p47phox, and p67phox, were detected in aortic tissue of SHR, but there was no significant effect of atorvastatin on the mRNA expression levels of these genes, as shown in Figure 5A. In agreement with the in vitro findings, rac1 GTPase protein expression was decreased in the membrane fraction of aortic homogenates of statin-treated SHR, whereas cytosolic rac1 protein expression was increased (n=3 per group), as demonstrated in a representative immunoblot in Figure 5B.

Finally, treatment with atorvastatin did not exert a significant effect on the vascular mRNA expression of the antioxidative enzymes mnSOD, ecSOD, GPX, and czSOD in vivo. However, consistent with the findings in VSMCs, vascular CAT mRNA expression was increased in the aortas of SHR treated with atorvastatin (157±15% of control, P<0.05 versus control; n=3 per group), as shown in Figure 5C.

Discussion

ROS such as superoxide and hydrogen peroxide are involved in the pathogenesis of atherosclerosis.17,18 This is based on a variety of effects exerted by ROS on vascular cells. Free radicals induce VSMC mitogenicity, endothelial dysfunction, endothelial cell apoptosis, and the adhesion of macrophages to endothelial cells; they also serve as intracellular signaling molecules and scavenge vasoprotective NO.17,18,29 One of the predominant sources of ROS is NAD(P)H oxidase, a multi-component enzyme system residing in white blood cells and in VSMCs and endothelial cells, the composition of which is dependent on the harboring cell type.19–24 Whereas, for example, gp91phox is expressed in endothelial cells and macrophages, nox1 substitutes gp91phox in VSMCs.23,24 Previous reports have suggested that the interaction of nox1 with other subunits is essential for the overall activity of...
NAD(P)H oxidase in VSMCs. In analogy, modulation of p22phox expression is also closely connected with NAD(P)H oxidase activity. Activation and translocation of rac1 GTPase from the cytosolic compartment to the cell membrane is another prerequisite of NAD(P)H oxidase activation, as shown in white blood cells. The role of other components of NAD(P)H oxidase, such as p40phox, p47phox, and p67phox, is less clear; however, the assembly of all subunits is required for superoxide production by this enzyme.

Given the importance of ROS production for atherosclerosis and the predominant role of NAD(P)H oxidase for free radical load in the vasculature, it is important to determine whether therapeutic interventions other than the blockade of the renin-angiotensin system may influence this multicomponent enzyme. Statins are drugs that are widely and successfully used in cardiovascular diseases; besides their lipid-lowering properties, these drugs demonstrate a variety of pleiotropic effects on vascular cells independent of plasma cholesterol levels. In endothelial cells and macrophages, it has been shown that statin-induced cellular depletion of intermediates of cholesterol biosynthesis can impair the action of rac1. Our data indicate that stimulation with angiotensin II leads to the enhancement of rac1 expression in the cell membrane, indicating that rac1 membrane translocation may be an important step for angiotensin II–induced ROS production in VSMCs. In contrast, statins decrease the geranylgeranylation-dependent translocation of rac1 from the cytosol to the cell membrane in the presence or absence of angiotensin II in these cells. This observation is extended to the in vivo situation, inasmuch as long-term statin treatment of SHR leads to a similar impairment of rac1 membrane translocation in the vessel wall. In this animal model, the expression levels of 2 other essential components of the NAD(P)H oxidase, namely, p22phox and nox1, were decreased by atorvastatin. These findings indicate that NAD(P)H oxidase is inhibited at various strategic points in vivo and suggest that the antioxidant properties of statins are mediated by the interaction with different genes engaged in the production of ROS in the vessel wall.

Nox1 expression was decreased in VSMCs treated with atorvastatin; this finding is consistent with the findings in SHR. Expression of p22phox was not affected in VSMCs. However, in cultured aortic endothelial cells, statin treatment led to a decreased mRNA expression of p22phox. This statin effect on endothelial cells may contribute to the decreased p22phox expression found in SHR aortas. Because the inhibitory effect of atorvastatin in endothelial cells is only moderate, the overall effect of the statin on p22phox expression in SHR cannot be completely explained by the in vitro findings in VSMCs and endothelial cells, indicating that in vivo other cofactors may participate in this statin effect.

The ultimate oxidative stress within vascular cells is determined by ROS production and corresponding elimination processes. The latter are realized by the radical-scavenging enzymes GPX, the SOD isoforms, and CAT. Whereas atorvastatin had no influence on the expression of GPX and SODs, CAT expression and activity were profoundly upregulated in vitro and in vivo. Physiologically, the upregulation of CAT can be observed after an increase of hydrogen peroxide concentrations. However, reduced superoxide production by NAD(P)H oxidase after decreased expression of essential subunits leads to reduced concentrations of hydrogen peroxide when SOD levels are not altered. The inhibitory effect of atorvastatin in endothelial cells is only moderate, the overall effect of the statin on p22phox expression in SHR cannot be completely explained by the in vitro findings in VSMCs and endothelial cells, indicating that in vivo other cofactors may participate in this statin effect.

The effects of atorvastatin on free radical production, nox1 and CAT expression, and membrane translocation of rac1 GTPase could be reversed by mevalonate but not by hydroxy-cholesterol, indicating that the statin effect is dependent on HMG-CoA reductase inhibition but not on reduced cholesterol synthesis and is, therefore, dependent on impaired isoprenylation processes.

It has been shown that statins upregulate ecNOS expression in endothelial cells. In VSMCs, ecNOS cannot be found, whereas the inducible isoform of NO synthase (iNOS) is expressed. iNOS produces only relevant concentrations of superoxide to hydrogen peroxide. Finally, this leads to a decrease of the overall intracellular free radical load in VSMCs. Therefore, upregulation of CAT may represent another antioxidant action of statins.
NO after induction by, for example, proinflammatory cytokines or lipopolysaccharides. In unstimulated VSMCs, as used in the present study, relevant levels of NO are not expected. Previous studies have demonstrated that statins may lead to an inhibition of iNOS induction and expression in blood vessels.\textsuperscript{34,35} Therefore, it seems unlikely that incubation with statins leads to increased NO levels, which could account for the decreased free radical load of VSMCs after statin treatment.

The aforementioned molecular effects of statins on rac1, p22phox, nox1, and CAT lead to reduced production of ROS in vitro and in vivo. It may be assumed that these mechanisms of action may participate in the well-established beneficial effects of statins on endothelial function.\textsuperscript{36,37} Inasmuch as a reduction of free radical load in the vasculature may lead to improved endothelium-dependent vasorelaxation and to reduced adhesion of macrophages to the vessel wall.\textsuperscript{17,18} Because ROS are involved in the pathogenesis of atherosclerosis,\textsuperscript{17,18,28} it may be speculated that reduced production and increased elimination of ROS within the vessel wall may resemble a pleiotropic cholesterol-independent effect of statins that may contribute to the atheroprotective properties of these drugs.

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References

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