HMG-CoA Reductase Inhibitors Regulate Inflammatory Transcription Factors in Human Endothelial and Vascular Smooth Muscle Cells

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Objective—Pleiotropic atheroprotective effects of HMG-CoA reductase inhibitors may be mediated on the level of vascular gene transcription. The aim of this study was to characterize the effects of statins on the activation of transcription factors known to regulate inflammation and cell proliferation/differentiation.

Methods and Results—Simvastatin, atorvastatin, and lovastatin (0.1 to 10 μmol/L) inhibited the binding of nuclear proteins to both the nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) DNA consensus oligonucleotides in human endothelial and vascular smooth muscle cells as assessed by electrophoretic mobility shift assay (EMSA). The inhibitory effects of statins on NF-κB or AP-1-dependent transcriptional activity were examined by transient transfection studies. HMG-CoA reductase inhibitors upregulated IκB-α protein levels in endothelial cells and decreased c-Jun mRNA expression in smooth muscle cells as analyzed by Western and Northern blotting, respectively. Furthermore, statins inhibited DNA binding of hypoxia-inducible factor-1α. Downstream effects of statins included inhibition of plasminogen activator inhibitor-1 and vascular endothelial growth factor-A mRNA levels in endothelial cells.

Conclusions—HMG-CoA reductase inhibitors downregulate the activation of transcription factors NF-κB, AP-1, and hypoxia-inducible factor-1α. These findings support the concept that statins have antiinflammatory and antiproliferative effects that are relevant in the treatment of atherosclerotic diseases. (Arterioscler Thromb Vasc Biol. 2003;23:58-63.)

Key Words: statins ■ nuclear factor-κB ■ activator protein-1 ■ hypoxia-inducible factor-1α ■ vascular endothelial growth factor

Randomized clinical trials have clearly shown the benefit of statin therapy in the reduction of cardiovascular events and total mortality in coronary heart disease patients with either high or normal cholesterol levels. In these studies, survival curves began to diverge within a relatively short period of time and before effects on plaque size were likely to occur. Demonstrated effects of HMG-CoA reductase inhibitors were not reflected by a regression in coronary stenoses as assessed by angiography. These findings have suggested that mechanisms of statins beyond lipid lowering are likely to be involved in the reduction of coronary events.

Both in vivo and in vitro studies support the notion that statins counteract the chronic subclinical vascular inflammatory state associated with atherosclerosis. Statins inhibit leukocyte-endothelium interaction and decrease inflammation in carotid lesions in humans. Many of the vasculoprotective effects of HMG-CoA reductase inhibitors seem to be mediated by enhanced availability of nitric oxide. There is increasing evidence that statins may act on the transcriptional level as well, e.g., simvastatin inhibited endothelial secretion of PAI-1, which was correlated with reduced mRNA transcription and activity of the promoter.

Despite extensive research on molecular mechanisms of statins, little is known about the interactions of these drugs with transcription factors. The aim of this study was to characterize the effects of simvastatin, atorvastatin, and lovastatin on the activation of nuclear factor (NF)-κB, activator protein (AP)-1, and hypoxia-inducible factor (HIF)-1α in endothelial and arterial smooth muscle cells. Because these factors regulate the transcription of many genes, including cytokines, chemokines, adhesion molecules, and growth factors, such interactions of statins on vascular cell signaling and gene expression may explain atheroprotective effects not directly related to cholesterol lowering.

Methods

Simvastatin (MSD) and lovastatin (Calbiochem) prodrugs were activated from their inactive lactone proforms to their active dihy-
Effects of Simvastatin, Atorvastatin, and Lovastatin on DNA Binding of Transcription Factor NF-κB, on NF-κB–Dependent Gene Expression in Human Endothelial and Vascular Smooth Muscle Cells, and on IκB-α Protein Levels in Human Endothelial Cells

As shown in Figure 1, simvastatin, atorvastatin, and lovastatin significantly inhibited basal NF-κB DNA binding activity (Ea.hy926 cells, lanes 1 through 6; human aortic endothelial cells, lanes 7 through 12; human aortic smooth muscle cells, lanes 13 through 17). As shown for simvastatin, this effect was already detected at doses of 0.1 μmol/L (lane 4). TNF-α–treated cells were used as a positive control (lanes 10 through 12), in which addition of antibodies directed against either p50 (lane 11) or p65 (lane 12) led to a marked supershift of the specific band (small arrow). Statins did not significantly affect nuclear protein binding to SP-1 consensus DNA sequences by 74.1%, 95.8%, and 92.3%, whereas SP-1 binding to consensus DNA sequences decreased only by 8.2%, 3.2%, and 13.5%, respectively.

Human endothelial cells (Ea.hy926) and aortic smooth muscle cells were transiently transfected with pNF-κB–SEAP vectors. Figure 2A shows SEAP activity in supernatants derived from Ea.hy926 cells 24 hours after incubation of 0.1 and 1 μmol/L simvastatin, atorvastatin, and lovastatin, as follows: control 100% (n=3); simvastatin 0.1 μmol/L 46.5% (n=3; SD±10.7; P=0.04); atorvastatin 0.1 μmol/L 53.0% (n=3; SD±3.9; P=0.04);lovastatin 0.1 μmol/L 98.5% (n=3; SD±27.4; P=NS) and control 100% (n=3); 1 μmol/L simvastatin 45.8% (n=3; SD±3.8; P=0.04); 1 μmol/L atorvastatin 0.1 μmol/L 33.5% (n=3; SD±13.9; P=0.04); and 1 μmol/L lovastatin 38.4% (n=3; SD±18.7; P=0.04).
In vitro incubation with 10 μmol/L simvastatin or 10 ng/mL TNF-α, as shown: control 100% (n=4; SD±7.2); 10 μmol/L simvastatin 67% (n=4; SD±6.6; P=0.02); TNF-α 230.5% (n=4; SD±17.5); and TNF-α+10 μmol/L simvastatin 156.5% (n=4; SD±23.9; P=0.02). Simvastatin and atorvastatin but not lovastatin (all at a concentration of 10 μmol/L) significantly decreased basal NF-κB activation also in human aortic smooth muscle cells, as shown in the left part of Figure 1B, as follows: control 100% (n=5; SD±13.4); 10 μmol/L simvastatin 69.6% (n=5; SD±12.9; P=0.008); 10 μmol/L atorvastatin 60.5% (n=5; SD±15.5; P=0.008); 10 μmol/L lovastatin 81.7% (n=5; SD±21.74; P=0.15).

Inhibition of NF-κB signaling by statins may be mediated by induction or stabilization of its cytosolic inhibitor IκB-α, because simvastatin, atorvastatin, and lovastatin increased IκB-α protein levels in Ea.hy926 cells within 2 hours of incubation (Figure 2B and data not shown).

Effects of Simvastatin, Atorvastatin, and Lovastatin on DNA Binding of Transcription Factor AP-1, AP-1–Dependent Gene Expression, and c-Jun mRNA Expression in Human Endothelial and Vascular Smooth Muscle Cells

The same nuclear extracts were additionally analyzed for binding to the AP-1 consensus sequence, as shown in Figure 3: atorvastatin, simvastatin, and lovastatin markedly decreased basal AP-1 DNA binding. This effect was dose-dependent and seen at concentrations >0.1 to 1 μmol/L, varying between different statins and cell types tested. In Ea.hy926 cells, atorvastatin profoundly diminished AP-1 activation at all doses tested (from 0.1 to 10 μmol/L; lanes 4, 5, 7, and 9), whereas lovastatin led to such an inhibition at concentrations >1 μmol/L (lane 3). Simvastatin showed weaker potency to inhibit AP-1 activation (lane 12), and concentrations >1 μmol/L of simvastatin were needed to inhibit AP-1 activation in human aortic endothelial cells (HAECs) (lanes 14 through 16). Addition of antibodies either led to a supershift (anti-JunD, lane 21, small arrow) or decreased DNA-binding capacity (anti-c-Fos, anti-c-Jun, anti-JunB, lanes 18 through 20). Simvastatin clearly inhibited LPS-stimulated AP-1 activation at concentrations of and above 0.5 μmol/L (lanes 22 through 28). Similar effects were seen in smooth muscle cells, in which simvastatin decreased basal AP-1 DNA binding already at 0.1 μmol/L (lanes 29 through 35). Densitometric analysis of lanes 10 through 12 showed that 0.1 and 1 μmol/L simvastatin decreased AP-1 binding to consensus DNA sequences by 11.8% and 35.2%, respectively.

The effects of statins on AP-1–dependent gene activation in endothelial cells were studied by transient transfection with pAP-1–SEAP vectors. Figure IA (available online at http://atvb.ahajournals.org) shows SEAP activity in supernatants derived from Ea.hy926 cells 24 hours after incubation with 10 μmol/L simvastatin, atorvastatin, and lovastatin, as follows: control 100% (n=5; SD±7.2); 10 μmol/L simvastatin 84.5% (n=5; SD±5.7; P=0.016); 10 μmol/L atorvastatin 72.4% (n=5; SD±8.1; P=0.009); and 10 μmol/L lovastatin 77.5% (n=5; SD±8.9; P=0.016). The effects of statins on AP-1–dependent gene activation in endothelial cells were studied by transient transfection with pAP-1–SEAP vectors. Figure IA (available online at http://atvb.ahajournals.org) shows SEAP activity in supernatants derived from Ea.hy926 cells 24 hours after incubation with 10 μmol/L simvastatin, atorvastatin, and lovastatin, as follows: control 100% (n=5; SD±7.2); 10 μmol/L simvastatin 84.5% (n=5; SD±5.7; P=0.016); 10 μmol/L atorvastatin 72.4% (n=5; SD±8.1; P=0.009); and 10 μmol/L lovastatin 77.5% (n=5; SD±8.9; P=0.016).
effects of statins at different concentrations on cell viability

A cytotoxicity assay based on LDH release was performed using the Promega CytoTox 96 assay. After 24 hours of incubation, there was no significant increase in LDH release, even when cells had been exposed to 10 μmol/L of statin treatment, as shown in Ea.hy926 cells as follows: control 100% (n=6); simvastatin 10 μmol/L 109.9% (n=6; SD±13.2); atorvastatin 10 μmol/L 110.3% (n=6; SD±7.3); and lovastatin 10 μmol/L 94% (n=6; SD±9.6). Similar results were obtained in HAECs and smooth muscle cells (data not shown). There was no sign of cytotoxicity after 24 hours of incubation of these experimental compounds (up to 10 μmol/L) as assessed by the MTT assay, as shown in Ea.hy926 cells (data not shown). In contrast, statins even at the lowest doses tested significantly decreased WST reduction, as shown in Ea.hy926 cells as follows: control 100% (n=6), simvastatin 0.1 μmol/L 74.84% (n=6; SD±5.43); simvastatin 1 μmol/L 64.83% (n=6; SD±5.16); simvastatin 5 μmol/L 70.47% (n=6; SD±7.71); simvastatin 10 μmol/L 70.84% (n=6; SD±4.7); atorvastatin 0.1 μmol/L 93.61% (n=6; SD±11.35); atorvastatin 1 μmol/L 81.47% (n=6; SD±8.58); atorvastatin 5 μmol/L 74.26% (n=6; SD±9.96); atorvastatin 10 μmol/L 74.27% (n=6; SD±10.1); lovastatin 0.1 μmol/L 89.89% (n=6; SD±13.99); lovastatin 1 μmol/L 82.83% (n=6; SD±9.25); lovastatin 5 μmol/L 92.72% (n=6; SD±13.51); and lovastatin 10 μmol/L 87.9% (n=6; SD±9.54). Decreased availability of superoxide anion whose formation is inhibited by statins may explain these findings.14 Reduction of WST but not of MTT with NADH is strongly inhibited by superoxide dismutase, indicating involvement of superoxide in the reductive mechanism.15 Trypan blue exclusion assays were performed at conclusion of most of the experiments and demonstrated >95% viability with no differences between control and statin-treated groups (data not shown).

Discussion

In this study, we show that statins inhibit the activation of transcription factors NF-κB, AP-1, and HIF-1α in cultured human endothelial and vascular smooth muscle cells. NF-κB has been recognized as one of the major transcription factors influencing key steps in the development of atherosclerotic lesions. NF-κB profoundly regulates the expression of a variety of genes entangled in vascular diseases, which mediate cell migration, promote inflammation, and control the balance between cell proliferation and apoptosis.16 The activation of NF-κB is involved in endothelial dysfunction and seems to be linked to its redox sensitivity.17 The latter phenomenon could explain the inhibitory effects of statins on NF-κB activation, because statins are able to scavenge oxygen-free radicals18 in addition to the stimulated endothelial nitric oxide production that inhibits NF-κB via the induction and stabilization of IκB-α.19 Activated NF-κB is present in human atherosclerotic plaque, whereas little activated NF-κB can be detected in vessels free of atherosclerosis.20 Patients with unstable but not with stable angina pectoris have high levels of activated NF-κB inuffy coat-derived cells,21 and data suggest that blockade of NF-κB in vivo prevents myocardial infarction.22 Previous studies showed conflicting results regarding the interaction of statins and NF-κB signaling. Atorvastatin reduces activation of transcription factor NF-κB in cultured vascular smooth muscle and mononuclear cells23 as well as in atherosclerotic...
lesions in the rabbit. Decreased NF-κB activity in mesangial cells has been detected on treatment with lovastatin, whereas simvastatin has been found to increase NF-κB activation in endothelial cells. Our investigations, being the first to use transient transfection techniques and to compare different statins at various concentrations, clearly support the notion that HMG-CoA reductase inhibitors curtail NF-κB signaling. In view of the role of NF-κB on inflammation and cell survival, the inhibition of basal as well as stimulated NF-κB activation may contribute to the anti-inflammatory, antiproliferative, and proapoptotic effects of statins.

Another transcription factor involved in the progression of atherosclerosis is AP-1, a collective term referring to dimeric proteins composed of Jun, Fos, or ATF (activating transcription factor) subunits. AP-1–regulated genes include matrix metalloproteinases, cytokines, chemokines, adhesion molecules, inducible nitric oxide synthase, cell cycle proteins, and Fas ligand. Our investigations show that statins markedly decrease basal vascular AP-1 activation. Furthermore, statins inhibited basal mRNA expression of c-Jun, which is a central component of all AP-1 complexes and whose expression is regulated by positive feedback mechanisms via AP-1 binding to the TPA responsive element within its promoter. Effects of statins on AP-1 DNA binding may be mediated at the level of small GTP proteins by induced farnesylation of Ras or geranylgeranylation of Rho. Ras leads to the activation of Raf, an initial kinase of the mitogen-activated protein kinase cascade, which results in AP-1 activation via phosphorylation of extracellular signal–regulated kinases. The observed inhibition of AP-1 signaling could explain some of the plaque-stabilizing effects of HMG-CoA reductase inhibitors, eg, decreased expression of metalloproteinases (MMPs) or PAI-1. Our findings are supported by previous studies showing that lovastatin inhibits platelet-derived growth factor–mediated and angiotensin II–mediated c-Jun and c-Fos expression in human smooth muscle cells and the proliferation of renal epithelial tubular cells through a p21ras–activated, AP-1–dependent pathway. Decreasing effects of statins on AP-1 signaling detected in transfection experiments were weaker compared with the EMSA findings. This might be explained by the fact that transcriptional cofactors may compensate some of the inhibition of AP-1 binding proteins as well that additional signaling pathways contribute to transcriptional activation, which are not affected by statins.

Hypoxia-inducible transcription factors (HIF-1α, HIF-1β, and HIF-2α) are now recognized as strongly influencing vascular gene transcription. The expression and activity of the HIF-1α subunit, which forms heterodimers with HIF-1β, are tightly regulated by cellular O2 concentrations. Under hypoxic conditions, HIF-1α activates the transcription of genes encoding erythropoietin, glucose transporters, glycolytic enzymes, VEGF and its receptors (VEGFR1, VEGFR2), and other genes whose protein products increase O2 delivery or facilitate metabolic adaption to hypoxia. HIF’s also induce the expression of genes involved in vascular tone, cell growth, and inflammation. However, the role of transcription factor HIF-1α in the pathogenesis of atherosclerosis is less well established. Therefore, the pathogenic implications of the inhibitory effect of statins on HIF-1α binding to the HRE must remain speculative. Acute hypoxia causes pulmonary vasoconstriction, and chronic hypoxia causes smooth muscle replication and extracellular matrix accumulation, resulting in vessel wall remodeling. Hypoxia suppresses both the transcriptional rate of the endothelial nitric oxide synthase gene and decreases the stability of its mRNA, effects that were blocked by statins. Many genes, such as PAI-1, endothelin-1, and platelet-derived growth factor-B, whose expression are downregulated on statin treatment, contain HRE elements in their promoter regions.

Effects of statins on signaling cascades may also be influenced by their profound effects on membrane fatty acid composition, eg, by a stimulated desaturation of linoleic acid (LA, 18:2 n-6) yielding increased arachidonic acid levels. Linoleic acid stimulates both NF-κB and AP-1 activation in vascular cells, whereas arachidonic acid decreases NF-κB signaling.

Our findings that statins inhibit HIF-1α signaling and decrease VEGF expression in vascular cells are supported by several recently published studies. Simvastatin attenuates hypoxia-mediated effects in coronary artery walls of pigs in experimental hypercholesterolemia, such as upregulation of HIF-1α, VEGF, MMP-2, and MMP-9 immunoreactivity. Cerivastatin inhibits endothelial cell migration, and plasma levels of VEGF in patients with hypercholesterolemia are decreased on treatment with fluvastatin as well as with atorvastatin. Such previously suggested antiangiogenic effects of statins should be considered for delaying atherosclerotic plaque growth but might also inhibit tumor progression. This has been supported by clinical studies that have demonstrated that statin treatment reduced the incidence of cancers. Whether statins decrease VEGF mRNA expression under normoxic conditions attributable to decreased binding of NF-κB, AP-1, or HIF-1α to the promoter remains to be investigated.

In conclusion, this study shows that statins profoundly influence the expression and activation of transcription factors. Such interactions may affect local gene transcription in atherosclerotic vessels and thereby inhibit inflammation, thrombosis, and cell proliferation. Additional studies are required to test whether statin treatment in CAD patients regulates gene transcription in a comparable manner in vivo.

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