Atorvastatin completely inhibits VEGF-induced ACE upregulation in human endothelial cells

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Atorvastatin completely inhibits VEGF-induced ACE upregulation in human endothelial cells. Am J Physiol Heart Circ Physiol 286: H2096-H2102, 2004. First published January 2, 2004; 10.1152/ajpheart.00894.2003.—Angiotensin-converting enzyme (ACE) plays an important role in the pathophysiology of cardiovascular disease. We investigated whether atorvastatin, a powerful agent for the prevention and treatment of cardiovascular disease, influences ACE production in endothelial cells. Human umbilical cord vein endothelial cells were treated with VEGF (476 pm), which induced ACE upregulation. Cotreatment with atorvastatin (0.1-10 μM) dose dependently inhibited VEGF-induced ACE upregulation. In the presence of mevalonate (100 μM), atorvastatin failed to downregulate VEGF-induced ACE production. Cotreatment of the cells with either farnesylpyrophosphate (FPP; 5 μM) or geranylgeranylpyrophosphate (GGPP; 5 μM) partially inhibited the suppressive effect of atorvastatin. Pretreatment of the cells with Rho-associated protein kinase inhibitor, Y-27632 (10 μM), partially inhibited VEGF-induced ACE upregulation. VEGF (476 pm) caused PKC phosphorylation, which was inhibited by cotreatment of the cells with atorvastatin. Atorvastatin inhibited VEGF-induced ACE upregulation probably by inhibiting PKC phosphorylation. This effect was mediated via inhibition of the mevalonate pathway. ACE downregulation may be an additional beneficial effect of statins in the treatment of cardiovascular disease.

Statins are effective drugs in the prevention of cardiovascular diseases like coronary heart disease and atherosclerosis (25). Statins act by blocking the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) to mevalonate, the precursor of cholesterol. Lowering of LDL cholesterol levels is associated with longer survival and lesser incidence of coronary disease. However, the beneficial effects of statins are extended to patients with normal cholesterol levels. Recent studies (25) suggest that statins have additional effects independent of LDL cholesterol lowering. By blocking HMG CoA, statins inhibit the synthesis of a variety of compounds derived from the mevalonate pathway, such as isoprenoids, which play a key role in many cellular processes. Isoprenoids are lipid attachments involved in posttranslational modification of some proteins, such as the small G proteins Ras and Ras-like proteins (14). In vitro studies show that statins inhibit proliferation of vascular smooth muscle cells (16) and endothelial cells (28), reduce adhesion of human monocytes to endothelial cells (26), decrease endothelin-1 production in endothelial cells (9), upregulate endothelial nitric oxide synthase in endothelial cells (14, 9), and downregulate AT-1 receptors in vascular smooth muscle cells (10).

Angiotensin-converting enzyme (ACE), a widely distributed enzyme on the luminal surface of vascular endothelium, catalyzes the proteolytic cleavage of ANG I to ANG II and has bradykinin-degrading activity (23). Thus ACE participates in the control of vascular resistance by generating ANG II and degrading bradykinin. ANG II also acts as a vascular growth factor participating in angiogenesis, vascular remodeling, and response to vascular wall injury and atherogenesis (31). As a regulator of ANG II production, ACE may have an important role in atherosclerosis and hypertension. Thus increased ACE accumulation in atherosclerotic blood vessel walls has been reported (6, 17). Furthermore, ACE inhibitors are effective both in reducing experimental atherogenesis (2) and in the reduction of left ventricular hypertrophy of hypertensive patients (4).

VEGF, a potent and specific mitogen for endothelial cells, is involved in several endothelium-specific functions such as migration, proliferation of endothelial cells, and angiogenesis (7). In addition to its physiological functions, VEGF also has a role in atherosclerosis and tumor growth (7). We have shown that VEGF is a potent stimulator of ACE in endothelial cells (22). In the present study, we investigated the mechanism by which atorvastatin inhibited VEGF-induced ACE upregulation in human endothelial cells.

MATERIALS AND METHODS

Endothelial Cell Culture

With the approval of the Committee of Ethics of the Department of Obstetrics and Gynecology, University of Helsinki, endothelial cells were prepared from human umbilical cord veins according to Jaffe et al. (12). Veins were cannulated, washed with PBS, and treated with 0.5% collagenase (Sigma; St. Louis, MO) in PBS for 15 min at room temperature, and cells were then collected by centrifugation. Cells were grown to confluence in 0.2% gelatin (Sigma)-coated cell culture flasks (Costar; Cambridge, MA) in medium 199 (GIBCO; Belmont, CA) supplemented with 20% FCS (GIBCO), 20 μg/ml endothelial cell growth supplement (Sigma), 12 μ/ml heparin (Sigma), 100 U/ml G-penicillin, 100 μg/ml streptomycin (GIBCO), and 2 mM l-glutamine (GIBCO) at 37°C in humidified 5% carbon dioxide in air. The cells were detached with 0.125% trypsin-0.02% Na2EDTA solution (GIBCO) and subcultured on 48-well cell culture plates (Costar) coated with 0.2% gelatin solution. The cells were identified as endothelial cells by their typical cobblestone appearance and the presence of von Willebrand factor demonstrated by an immunofluorescence method using rabbit immunoglobulin to human von Willebrand factor.
Confluent subcultures at passages 1 and 2 were incubated in medium 199 supplemented with 5% FCS. Cell cultures were incubated for 24 h with or without the following substances: atorvastatin (0.1–10 μM), VEGF165 (476 pM), mevalonate (100 μM), farnesylpyrophosphate (FPP; 5–10 μM), geranylgeranylpyrophosphate (GGPP; 5–10 μM), or (R)-()+-trans-N-(4-pyridyl)-4-(1-aminoethoxy)-cyclohexanecarboxamide dihydrochloride (Y-27632; 10 μM). Cells were preincubated for 30 min with atorvastatin or Y-27632 before VEGF was added and then incubated for further 24 h. Mevalonate, GGPP, or FPP was added at the same time as atorvastatin. After incubation time, ACE assay was performed as described below. Atorvastatin, and Y-27632 were from Calbiochem, San Diego, CA; other substances were from Sigma.

The effect of test substances on cellular viability and growth were tested by a CellTiter 96-cell proliferation/cytotoxicity assay kit (Promega; Madison, WI) and by a bichinchoninic acid protein assay kit (Pierce; Rockford, IL).

ACE Inhibitor Binding Assay

ACE amount in intact endothelial cells was measured by an inhibitor-binding assay developed and characterized in our laboratory (21). Briefly, a lisinopril analog, p-hydroxybenzamidine derivative of N-(1-carboxy-3-phenylpropyl)-L-lysyl-L-proline (351A; Merck, Sharp and Dohme; Rahway, NJ), was labeled with 125I (IMS 30; Amersham; Buckinghamshire, UK) using the chloramine T method, according to the manufacturer’s instructions. Brieﬂy, cells were rinsed with ice-cold PBS and lysed with SDS sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue and immediately scraped off the plate. An aliquot of sample was boiled for 5 min, centrifuged and applied to SDS-PAGE gel for Western blot analysis. Equal amounts of protein in the supernatants were subjected to SDS-PAGE. A 10% SDS-PAGE bis-acrylamide gel was run at 100 v for 1.5 h. After transfer to nitrocellulose membranes, the membranes were blocked overnight with primary antibody, phospho-PKC, or PKC antibody (1:1,000) and the membranes were incubated with horseradish peroxidase-conjugated secondary antibody 1:2,000 (Cell Signaling Technology). The bands were then visualized with Western Blot Chemiluminescence Reagent Plus (New England Nuclear Life Science Products; Boston, MA) after autoradiography, according to manufacturer’s instructions. Bands were quantiﬁed by Science Lab 99 Image Gauge software (Fuji Photo Film).

Statistical Evaluation

Results are expressed as means ± SD of eight replicate determinations from three to four separate experiments. ANOVA followed by Bonferroni’s multiple-comparison test was applied.

RESULTS

Effect of Atorvastatin on VEGF-Induced ACE Upregulation

Atorvastatin at doses of 0.1–10 μM had no signiﬁcant effect on basal ACE production measured after 24-h treatment (Fig. 1). Cotreatment of endothelial cells with atorvastatin (0.1–10 μM) dose dependently inhibited VEGF (476 pM)-induced ACE upregulation (Fig. 1).

Modulation of Mevalonate on the Suppressive Effect of Atorvastatin

To conﬁrm that the effect of atorvastatin on VEGF-induced ACE was mediated by inhibition of HMG CoA reductase, HUVECs were coincubated with VEGF (476 pM), atorvastatin (10 μM), and mevalonate (100 μM), the direct metabolite of HMG CoA reductase. Atorvastatin failed to downregulate
VEGF-induced ACE production in the presence of mevalonate, suggesting that the mevalonate pathway indeed was involved (Fig. 2). Mevalonate (100 μM) did not modulate basal ACE amount (data not shown).

Modulation of Isoprenoids, FPP, and GGPP on the Suppressive Effect of Atorvastatin

We then studied whether the suppressive effect of atorvastatin on VEGF-induced ACE was due to inhibition of the isoprenoid intermediates FPP and GGPP. Cotreatment of the cells with either FPP (5 μM) or GGPP (5 μM) and atorvastatin partially inhibited the suppressive effect of atorvastatin on VEGF-induced ACE. Higher concentrations of FPP (10 μM) or GGPP (10 μM) were not more effective (data not shown). Cotreatment of the cells with both FPP (5 μM) and GGPP (5 μM) and atorvastatin completely reversed the suppressive effect of atorvastatin on VEGF-induced ACE production (Fig. 2). These data suggest the involvement of both FPP and GGPP in VEGF-induced ACE upregulation.

Effect of Atorvastatin on VEGF-Induced ACE mRNA Level

To study whether the suppressive effect of atorvastatin on VEGF-induced ACE upregulation was also found at mRNA level, real-time PCR was used to quantify the levels of ACE mRNA and 28S rRNA. Single and sharply defined melting curves with narrow peaks were obtained for both PCR products. Once the predicted lengths of the PCR products were confirmed by agarose gel electrophoresis the melting temperature was used to identify specific products in subsequent analysis. Levels of the 28S rRNA did not vary significantly between treatments, whereas atorvastatin treatment caused inhibition of VEGF-induced ACE mRNA levels (Fig. 3).

Effect of Rho-Associated Protein Kinase Inhibitor Y-27632 on VEGF-Induced ACE Upregulation

Involvement of Rho proteins in VEGF-induced ACE upregulation was then studied by using the selective Rho-associated protein kinase inhibitor Y-27632. Pretreatment of the cells with Y-27632 (10 μM) partially inhibited VEGF-induced ACE upregulation, suggesting that Rho proteins were involved in ACE upregulation (Fig. 4).

Inhibition of VEGF-Induced PKC Phosphorylation by Atorvastatin

Treatment of HUVECs with VEGF (476 pM) induced PKC phosphorylation measured after 15-min stimulation that was inhibited by preincubation of the cells for 30 min with atorva-
statin (10 μM). Nonphosphorylated PKC levels were not significantly modulated by treatments (Fig. 5).

Growth or Toxicity Effects

None of the test substances incubated with confluent endothelial cell cultures had toxic or growth effects as tested by a CellTiter 96-cell proliferation/cytotoxicity assay kit or by bicinchoninic acid protein assay kit (data not shown).

DISCUSSION

We show here that atorvastatin, an inhibitor of HMG-CoA reductase, inhibited VEGF-induced ACE upregulation at both
protein and mRNA levels in endothelial cells. The effect of atorvastatin was reversed by mevalonate, the direct metabolite of HMG-CoA reductase. This confirmed that the effect of atorvastatin was mediated via the mevalonate pathway.

Role of Isoprenoid Intermediates

Statins are potent inhibitors of cholesterol synthesis. It has been assumed that lowering of cholesterol level is the sole mechanism underlying the beneficial effects of statins on cardiovascular diseases. However, recent experimental and clinical evidence indicates that the effect of statins may extend beyond their ability to reduce serum cholesterol level (25). Many of these cholesterol-independent effects are mediated by inhibiting the synthesis of the isoprenoid intermediates FPP and GGPP, which serve as lipid attachments for the superfamily of Ras GTPases (25). FPP and GGPP are essential for membrane attachment and biological activity of Ras and Rho, respectively. Inhibition of Ras and Rho isoprenylation lead to the accumulation of inactive Ras and Rho in the cytoplasm. Rho proteins are mediators of various intracellular processes. These proteins are linked to activation, contraction, or proliferation of vascular cells (19). Inhibition of Rho isoprenylation mediates many of the reported cholesterol-independent effects of statins in vascular wall cells like endothelial NOS stimulation (14), AT-1 receptor downregulation (10), and upregulation of cyclooxygenase-2 expression (5). We show here that the suppressive effect of atorvastatin on VEGF-induced ACE was partially reversed by FPP or GGPP and totally reversed by cotreatment with these isoprenoids. This suggests that Ras and Rho proteins are involved in VEGF-induced ACE upregulation. The involvement of Rho proteins in VEGF-induced ACE upregulation was further supported by the finding that the Rho-associated protein kinase inhibitor Y-27632 partially inhibited VEGF-induced ACE production.

Role of PKC

An association between Rho GTPases and PKC has been suggested. A recent study (24) shows a direct protein-protein interaction of PKC with Rho GTPases that results in kinase activation.

PKC is an intracellular signaling molecule involved in ACE regulation. Thus activation of PKC with phorbol ester (PMA), caused a marked increase of ACE (22, 27). We have previously reported that PKC is a mediator involved in VEGF-induced ACE production. Inhibition of PKC by the selective PKC inhibitor GF-109203X or downregulation of PKC totally blocked VEGF-induced ACE upregulation (22). Therefore, we studied whether PKC phosphorylation was modulated by atorvastatin. VEGF was shown to induce PKC phosphorylation that was inhibited by atorvastatin. We hypothesize that atorvastatin, by inhibiting Rho isoprenylation, may interfere with the interaction between Rho and PKC and thus inhibits VEGF-induced PKC phosphorylation.

Interaction of VEGF and ACE: Relevance for Atherosclerosis

ACE and VEGF are both thought to play important roles in the pathophysiology of cardiovascular diseases. VEGF, a potent and specific mitogen for endothelial cells, is postulated to be the major growth factor responsible for angiogenesis. Angiogenesis, the growth of new blood vessels, is needed for...

Fig. 4. Effect of (R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexancarboxamide dihydrochloride (Y-27632) on VEGF-induced ACE upregulation in HUVEC after 24-h incubation. Y-27632 (10 μM) was added 30 min before VEGF (476 pM). Bars indicate means ± SD. Significant differences were as follows: ***P < 0.001 vs. control and †††P < 0.001 vs. VEGF.

Fig. 5. Effect of atorvastatin (10 μM) on VEGF-induced PKC phosphorylation in HUVECs. Confluent HUVECs were treated with VEGF (476 pM) for 15 min in the absence or presence of atorvastatin (10 μM). Atorvastatin was added 30 min before VEGF. Cell extracts were prepared and subjected to SDS-PAGE and immunoblotted with phospho-PKC or PKC antibody. A: PKC and phospho-PKC gel bands from a typical experiment. B: bars indicate quantification of the bands by Science Lab 99 Image Gauge software. Bars are relative phospho-PKC levels normalized to total PKC. Bars indicate means ± SD. Significant differences were as follows: ***P < 0.001 vs. control and †††P < 0.001 vs. VEGF.
physiological functions but also occurs in pathological conditions, such as atherosclerosis, diabetic retinopathy, and tumor growth (7). The renin-angiotensin system plays a role in many cardiovascular disorders in which angiogenesis is induced. This includes myointimal proliferation after vascular injury, atherosclerosis, and diabetic angiopathy. Association of enhanced vascular ACE expression with the development of coronary atherosclerosis in humans has been demonstrated (6, 17). Furthermore, ACE inhibitors have vasculoprotective effects, which may contribute to the prevention of coronary atherosclerosis (2, 4). Several studies in hypercholesterolemic animal models have demonstrated that ACE inhibitors attenuate the development of atherosclerosis (13). On the other hand, induction of VEGF in human atherosclerotic lesions and in animal models of arterial injury has been described (3, 20). Therefore, the synergistic interaction between VEGF and ACE may enforce detrimental growth processes in the vascular wall. Statins represent one of the most powerful classes of agents for the treatment and prevention of cardiovascular disease. Statins appear to have beneficial effects on the vascular wall independent of their cholesterol-lowering effects. These actions include nitric oxide-dependent improvement of endothelial function, antioxidant effects, anti-inflammatory properties, and stabilization of atherosclerotic plaques (25). It has been shown that statins have antiangiogenic effects associated with proliferative activity of angiogenic factors like VEGF (28, 30). VEGF as a proangiogenic agent increases plaque neovascularization and progression (1). It has been suggested that the benefit of statin therapy on the progression of atherosclerosis is due, in part, to inhibition of plaque neovascularization (28). Our study suggests that statin therapy may have an additional benefit by inhibiting VEGF-induced ACE upregulation. In addition to statins, ACE inhibitors are effective in the prevention and treatment of atherosclerosis. Furthermore, combination of statins and ACE inhibitors may have additive or synergistic beneficial effects to prevent the development of atherosclerosis (18). A study (15) on rats with experimental cardiac hypertrophy suggested that simvastatin reduced left ventricular hypertrophy, in part, by reducing cardiac tissue ACE activity. Accordingly, our study suggests that statins may also have ACE inhibiting effects, although these in vivo results cannot be directly adapted to in vivo situations.

In conclusion, atorvastatin completely inhibited VEGF-induced ACE upregulation in endothelial cells probably by inhibiting PKC activation. This effect was mediated via the mevalonate pathway, and inhibition of both FPP and GGPP were involved. Because FPP and GGPP are essential for the activity of small G proteins Ras and Rho, inhibition of these proteins may constitute the mechanism by which atorvastatin inhibits ACE upregulation. The finding that atorvastatin inhibited VEGF-induced ACE upregulation may be an additional beneficial effect of statins in the prevention and treatment of cardiovascular disease.

GRANTS

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REFERENCES


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