Atorvastatin inhibits angiotensin-converting enzyme induction in differentiating human macrophages

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Saijonmaa O, Nyman T, Fyhrquist F. Atorvastatin inhibits angiotensin-converting enzyme induction in differentiating human macrophages. Am J Physiol Heart Circ Physiol 292: H1917–H1921, 2007. First published December 8, 2006; doi:10.1152/ajpheart.00920.2006.—Statins are effective drugs in the prevention of cardiovascular disease. Recent studies suggested that statins have additional beneficial effects on the vascular wall independent of their cholesterol-lowering effects. We investigated whether atorvastatin influences angiotensin-converting enzyme (ACE) production in differentiating human macrophages. Human peripheral blood monocytes (PBM) were isolated from fresh buffy coats. The cells were allowed to differentiate for 0–8 days in macrophage serum-free medium with 5 ng/ml granulocyte-macrophage colony-stimulating factor. Atorvastatin (0.005–0.5 μM), mevalonate (200–400 μM), geranylgeranyl pyrophosphate (1.25–2.5 μM), and/or farnesyl pyrophosphate (FPP; 1.25–2.5 μM) was added on the second day of differentiation and then every other day. After incubation time, the ACE amount in intact macrophages was measured. ACE amount in PBM was low. A marked time-dependent ACE induction was noticed during differentiation of monocytes to macrophages. Atorvastatin treatment inhibited ACE induction during differentiation. In the presence of mevalonate, atorvastatin failed to downregulate ACE production. Cotreatment of the cells with atorvastatin and FPP reversed the suppressive effect of atorvastatin on ACE. In conclusion, atorvastatin inhibited ACE upregulation, normally occurring in differentiating human macrophages. This effect was mediated via the mevalonate pathway, and inhibition of FPP was probably involved. The finding that atorvastatin inhibited ACE upregulation may represent a novel pleiotropic action and an additional beneficial effect of statins in treatment of cardiovascular disease.

METHODS

Cell Culture

Fresh buffy coats from healthy 18- to 65-yr-old blood donors were obtained from The Finnish Red Cross blood service (Helsinki, Finland). Each buffy coat (50 ml) was diluted 1:3 in PBS and layered beneath a density gradient (Ficoll-Pague; Pharmacia Biotech, Uppsala, Sweden). The gradient was centrifuged at 400 g for 20 min, and cells at the gradient interface were recovered and washed three times in PBS. The cells were then resuspended in 50 ml of macrophage serum-free medium (SFM; GIBCO Laboratories, Belmont, CA) containing 100 U/ml G-penicillin and 100 μg/ml streptomycin (GIBCO) and 2 mM l-glutamine (GIBCO). The cells were counted and plated at a density of 5.5 × 10⁴ cells/well in 24-well plates (Becton Dickinson, Franklin Lakes, NJ) where only monocytes are adherent. Monocytes were allowed to attach to the wells for 60 min at 37°C in 5% CO₂, after which nonadherent cells are removed by washing three times with PBS. The cells were then cultured in macrophage-SFM with 5 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF; Sigma Chemical, St. Louis, MO) to induce monocyte differentiation. For RNA and PKC experiments, cells were plated on six-well plates.

Experimental Design

To study ACE production during differentiation of human monocytes to macrophages, we cultured cells in macrophage-SFM supplemented with 5 ng/ml GM-CSF for 0–8 days. To study the effect of

Statins are effective drugs in the prevention of cardiovascular diseases such as coronary heart disease and atherosclerosis (15). 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 have suggested that statins have additional effects independent of LDL cholesterol lowering. In vitro studies show that statins inhibit proliferation of vascular smooth muscle cells (11) and endothelial cells (19), reduce adhesion of human monocytes to endothelial cells (16), decrease ET-1 production in endothelial cells (6), upregulate endothelial nitric oxide synthase in endothelial cells (6, 10), and downregulate ANG II type 1 receptors in vascular smooth muscle cells (7). Recently, we have shown that atorvastatin inhibited VEGF-induced ACE upregulation in human endothelial cells (14). In the present study we investigated the effect of statins on ACE induction in differentiating human macrophages.

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atorvastatin on ACE induction during macrophage differentiation, we treated cell cultures with atorvastatin at the beginning of the maturation period and every 2 days thereafter. In some experiments, cell cultures were coincubated with atorvastatin (0.5 µM) and mevalonate (200–400 µM), farnesylpyrophosphate (FPP; 1.25–2.5 µM), or geranylgeranyl pyrophosphate (GGPP; 1.25–2.5 µM). After incubation, an ACE assay was performed as described below. Atorvastatin was obtained from Calbiochem (San Diego, CA); other substances were obtained from Sigma. The effect of test substances on cellular viability and growth was tested using a CellTiter 96-cell proliferation/cytotoxicity assay kit (Promega, Madison, WI) and a bicomchonic acid (BCA) protein assay kit (Pierce, Rockford, IL).

ACE Inhibitor Binding Assay

The amount of ACE in intact cell cultures was measured using an inhibitor binding assay (IBA) developed and characterized in our laboratory (12). Briefly, the lisinopril analog 351A [p-hydroxybenzamidine derivative of N-(1-carboxy-3-phenylpropyl)-l-lysyl-l-proline; Merck, Sharp and Dohme, Rahway, NJ] was labeled with 125I (IMS 30; Amer sham, Amersham, UK) according to the chloramine T method, as described elsewhere (12). 125I-351A is specifically bound to ACE (4, 8). At the end of the incubation period with test substances, the conditioned medium was removed and the label was added in excess at 50,000 cpm per well. Following incubation at 37°C for 2 h, cells were washed with PBS, detached with 0.1 M NaOH, and counted in a gamma counter. The amount of ACE is given as the amount of inhibitor (125I-351A) bound (in cpm/mg protein). The amount of ACE inhibitor bound is proportional to the amount of ACE on the cell membrane (12). The method was previously shown to correspond to an enzyme activity method both in our laboratory (4) and elsewhere (20).

ACE mRNA Measurement

ACE mRNA was measured from macrophages allowed to differentiate for 8 days with or without atorvastatin (0.5 µM) treatment. Atorvastatin was added at the beginning of the maturation period and every 2 days thereafter. In some experiments cell cultures were coincubated with atorvastatin (0.5 µM) and mevalonate (400 µM) or FPP (1.25–2.5 µM).* Total RNA and cDNA preparation. Total RNA from macrophages was isolated using the GenElute mammalian total RNA kit (Sigma) according to the manufacturer’s instructions. After DNase I treatment (Sigma), RNA concentration was measured using the RiboGreen fluorescent nucleic acid stain (RNA quantification kit; Molecular Probes, Eugene, OR) and checked by agarose gel electrophoresis. The 0.5 µg of total RNA was transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Life Technologies, Pais ley, UK) and oligo (dT)12–18 (500 µg/ml) primer.

Quantification of ACE mRNA and 28S rRNA. Quantification of the mRNAs was performed using real-time PCR with LightCycler technology (Roche Diagnostics, Mannheim, Germany). Two microliters of cDNA diluted 1:10 were brought to a final volume of 20 µl, containing 4 mM MgCl₂, 2 µl of LightCycler-FastStart DNA SYBR Green I mix (Roche Diagnostics), and 0.5 µM of primers in H₂O. After initial activation of the DNA polymerase at 95°C for 10 min, the amplification conditions were as follows: 40 cycles consisting of denaturation for 15 s at 95°C, annealing for 5 s at 54°C (28S rRNA) or 3 s at 56°C (ACE), and extension at 72°C. The extension times (in s) were calculated from the amplicon size (base pairs/25). Fluorescent data were acquired at the end of each extension phase. After amplification, a melting curve analysis from 65 to 95°C with a heating rate of 0.1°C/s with a continuous fluorescence acquisition was completed. The primers used for 28S rRNA (accession no. X00525) were forward, 5'-TTG AAA ATC CGG GGG AGA G, and reverse, 5'-ACA TTG TTC CAA CAT GCC AG (amplicon size 100 bp), and primers for ACE (accession no. AF118569) were forward, 5'-ACC AAT GAC ACG GAA AG, and reverse, 5'-GTG GGT TTC GTT TC TC TG (amplicon size 207 bp). Standard curves were created from specific PCR products. The signals of the samples of interest were then quantified from the standard curve, and the expression of ACE was determined relative to 28S rRNA.

Immunoblotting of Phosphorylated PKC

Monocytes were allowed to differentiate for 8 days without or with atorvastatin (0.5 µM). Atorvastatin was added at the beginning of the maturation period and every 2 days thereafter. Cells were harvested for immunoblotting after 2, 5, and 8 days of differentiation. Phosphorylated and nonphosphorylated PKC levels were measured using phospho-PKC (pan) antibody (Cell Signaling Technology, Beverly, MA) and PKC (MC5) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Western blot analysis was performed according to the manufacturer’s instructions (Cell Signaling Technology). Briefly, cells were rinsed with ice-cold PBS, lysed with PBS containing 25.6 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromophenol blue, and immediately scraped off the plate and sonicated. An aliquot of sample was boiled for 5 min, centrifuged, and applied to SDS-PAGE gel for Western blotting. Equal amounts of protein in the supernatants were subjected to SDS-polyacrylamide gel electrophoresis. A 10% SDS-PAGE bis-acrylamide gel was run at 100 V for 1.5 h. After transfer to nitrocellulose membranes, the membranes were blotted overnight with the primary antibodies (phospho-PKC or PKC antibody, 1:1,000), and then the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000; Cell Signaling Technology). The bands were visualized with Western blot chemiluminescence Reagent Plus (NEN Life Science Products, Boston, MA) following autoradiography according to manufacturer’s instructions. Bands were quantified using Science Lab 99 Image gauge software (Fuji Photo Film).

Statistical Evaluation

Results are expressed as means (SD) of four replicate determinations from three to five separate experiments. Each experiment was performed using cells from a separate blood donor, and the results were normalized to control values of each experiment. ANOVA followed by Bonferroni’s multiple-comparison test was applied.

RESULTS

Effect of Atorvastatin on ACE Induction During Macrophage Differentiation

Peripheral blood monocyte (PBM) cultures were allowed to differentiate for 0–8 days. The differentiation of the cells could be seen simultaneously as typical morphological changes that included growth of cell size, increase in granularity, cell clustering, and development of spindle-shaped cells. The ACE amount on isolated PBM was low. During differentiation, membrane-bound ACE levels increased. Simultaneous treatment with atorvastatin did not significantly decrease ACE production during the first days of differentiation but inhibited ACE upregulation at the end of the maturation period (Fig. 1). Protein levels increased during differentiation, but no significant differences were detected between controls and atorvastatin-treated cells. After 2 days of differentiation, protein concentrations were 143 (SD 11) and 138 (SD 12) µg/ml in controls and atorvastatin (0.5 µM)-treated cells, respectively. Corresponding values after 5 days were 229 (SD 19) and 204 (SD 24) µg/ml and after 8 days, 258 (SD 20) and 243 (SD 15) µg/ml. Inhibition of ACE induction by atorvastatin (0.005–0.5 µM) was dose dependent (Fig. 2). Inhibition of ACE induction

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also was observed using simvastatin, suggesting a group effect (unpublished results from our laboratory). Morphological changes occurring during macrophage differentiation and the number of cells were similar between control and atorvastatin-treated cells.

Modulation of Mevalonate on the Suppressive Effect of Atorvastatin

To study whether the effect of atorvastatin on ACE was mediated by inhibition of HMG-CoA reductase, we cotreated cell cultures with atorvastatin (0.5 μM) and mevalonate (200–400 μM), the direct metabolite of HMG-CoA reductase. Atorvastatin failed to downregulate ACE production in the presence of mevalonate (400 μM), suggesting that the mevalonate pathway was involved (Fig. 3).

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

We then studied whether the suppressive effect of atorvastatin on ACE production was due to inhibition of the isoprenoid intermediates FPP or GGPP. Cotreatment of the cell cultures with FPP (1.25 μM) and atorvastatin (0.5 μM) reversed the inhibitory effect of atorvastatin on ACE production, whereas cotreatment with GGPP (1.25–2.5 μM) was without effect (Fig. 4).
Effect of Atorvastatin on PKC Phosphorylation

PKC phosphorylation was increased during macrophage differentiation. Atorvastatin treatment did not significantly affect PKC phosphorylation measured after 2, 5, or 8 days of differentiation (Fig. 5). Nonphosphorylated PKC levels were not significantly modulated during differentiation.

Effect of Atorvastatin on ACE mRNA Levels

To study whether the suppressive effect of atorvastatin on ACE upregulation also could be found at mRNA level, we used real-time PCR 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 ACE mRNA levels, an effect that was reversed by coincubation with mevalonate (400 μM) or FPP (1.25 μM) (Fig. 6).

DISCUSSION

We have shown that atorvastatin, an inhibitor of HMG-CoA reductase, inhibited ACE induction in differentiating human macrophages at both mRNA and protein levels. An effective atorvastatin concentration was 0.005 μM. In patients treated with atorvastatin, serum atorvastatin concentration varies between 0.002 and 0.2 μM (1). Statin concentrations >1 μM have been reported to arrest the functional differentiation of monocytes into macrophages (17). We did not observe reduced cell numbers or morphological differences between control and atorvastatin-treated cell cultures at statin concentrations used in the present study. Because atorvastatin-caused inhibition of ACE induction was also measured at mRNA level, we suggest that ACE reduction in cell membrane was a result of reduced ACE production, rather than interference with ACE trafficking to the cell surface or shedding.

The inhibitory effect of atorvastatin on ACE induction was reversed by coincubation of the cell cultures with mevalonate. This observation confirmed the specific statin effect and a role of the mevalonate-isoprenoid pathway in ACE induction.

Recent experimental and clinical evidence indicates that the effect of statins may extend beyond their ability to reduce serum cholesterol level. By blocking HMG-CoA, statins inhibit the synthesis of a variety of compounds derived from the mevalonate pathway, which play a key role in many cellular processes. Thus statins probably also exert their cardiovascular benefits via direct antiatherogenic properties in the arterial wall. Those beneficial effects include nitric oxide-dependent improvement of endothelial function, antioxidant effects, and anti-inflammatory properties and stabilization of atherosclerotic plaques (15).

Many of these cholesterol-independent effects of statins are mediated by inhibiting the synthesis of the isoprenoid intermediates FPP and GGPP, which serve as lipid attachments for the superfamily of Ras GTPases (15). We have earlier reported that atorvastatin downregulated VEGF-induced ACE upregulation in cultured human endothelial cells and that inhibition of the isoprenoid intermediates FPP and GGPP were involved. The present study suggests that in macrophages, only FPP is involved in ACE regulation.

In human endothelial cells, PKC is an intracellular signaling molecule involved in ACE induction (18, 13). We have shown previously that atorvastatin inhibited VEGF-induced ACE upregulation in human endothelial cells, probably by inhibiting PKC phosphorylation (14). PKC activation is implicated in induction of macrophage differentiation (9). In the present study we have shown an increase of PKC phosphorylation during macrophage differentiation that was not significantly inhibited by atorvastatin. This finding suggests that inhibition of ACE induction by atorvastatin was not due to PKC inhibition. The above-discussed observations point to differences in intracellular signaling mechanisms depending on cell origin.

Numerous clinical and laboratory studies have shown the relevance of the renin-angiotensin system in the pathogenesis of atherosclerosis. ANG II, the main effector peptide of renin-angiotensin system, has an important role in the pathogenesis of the renin-angiotensin system.
of atherosclerosis, for instance, by stimulation of macrophage-mediated LDL oxidation (21) and stimulation of the biosynthesis of various growth factors that are important in the development of atherosclerosis (21).

ACE contributes to the progression of atherosclerosis via an increase in vascular ANG II formation and inactivation of bradykinin, a potent antiatherogenic substance (2). Association of enhanced vascular ACE expression with the development of coronary atherosclerosis in humans has been demonstrated (3). In atherosclerotic lesions, marked ACE expression is located in macrophages. Studies with ACE knockout mice show that tissue ACE deficiency leads to a reduction of both oxidative stress and atherosclerosis (5), underlining the role of tissue ACE in atherogenesis. ACE inhibitors have vasculoprotective effects in human and animal models that may contribute to the prevention of coronary atherosclerosis (2).

Macrophages are instrumental to both atherogenesis and the progression of atherosclerotic lesions. The present results suggest that atorvastatin may have beneficial antiatherogenic effects by inhibiting ACE induction in differentiating human macrophages.

In conclusion, atorvastatin inhibited ACE induction occurring during macrophage differentiation. The effect of atorvastatin was mediated via the mevalonate pathway, and inhibition of FPP was involved. The finding that atorvastatin inhibited ACE induction during macrophage differentiation may represent a novel pleiotropic action and an additional benefit of statins in the prevention and treatment of atherosclerosis.

GRANTS

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