The Effect of Statins on mRNA Levels of Genes Related to Inflammation, Coagulation, and Vascular Constriction in HUVEC

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Large-scale clinical trials have demonstrated significant reductions in cardiovascular events following statin therapy. The observed benefit of statin therapy, however, may be greater in these trials than is to be expected from lowering lipid levels alone. In order to clarify the mechanism by which statins prevent cardiovascular events in vascular wall cells, we investigated the changes in gene expression profiles after incubation with atorvastatin or pitavastatin in cultured human umbilical vein endothelial cells using DNA microarrays. Statins affected the expression levels of genes involved in inflammation, coagulation, and vascular constriction. The mRNA levels for interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) decreased after statin treatment. Statins reduced mRNA levels of plasminogen activator inhibitor-1 (PAI-1) and increased the mRNA levels of thrombomodulin. Statins reduced the mRNA levels of endothelin-1 and increased the mRNA levels of nitric oxide synthase-3 (eNOS). These results show that, statins are clinically effective because of their ability to change the gene expression profile of endothelial cells thereby preventing vascular events. *J Atheroscler Thromb*, 2002; 9: 178-183.

Key words: HMG-CoA reductase, Statin, Pleiotropic effects, HUVEC

Introduction

Statins inhibit 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase and the synthesis of cholesterol, which leads to the upregulation of the low-density lipoprotein (LDL) receptor and improves the survival of individuals at risk for atherosclerotic disorders (1,2). Recently, many pleiotropic effects of statins have been reported. Statins decrease smooth muscle cell migration and proliferation. (3), decrease pre-pro endothelin-1 (ET-1) mRNA

expression (4), and augment basal nitric oxide dilator functions of the endothelium (5). HMG-CoA reductase inhibitors increase the expression of endothelial nitric oxide synthase by blocking Rho geranylgeranylation (6).

Pitavastatin (NK-104) ((+)-monocalcium bis [(3R, 5S, 6E)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoate]), which was synthesized as a quinoline derivative, has shown total cholesterol and triglyceride-lowering effects in beagle dogs and guinea pigs and has completed phase III clinical studies in Japan (7,8). In clinical studies, pitavastatin was able to lower low-density lipoprotein cholesterol levels by 33.4% (1 mg/day for 12 weeks) to 54.4% (8 mg/day for 12 weeks) and has a greater triglyceride-lowering effect than currently available inhibitors of HMG-CoA reductase (9). Pitavastatin increases the expression of Bone morphogenetic protein-2 and osteocalcin in primary cultured human osteoblasts and reduces osteopontin expression in rat aortic smooth

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muscle cells (10, 11). Among various inhibitors of HMG-CoA reductase, pitavastatin most effectively induced the expression of the LDL receptor mRNA at between 0.1 and 10 μ M. When cells were treated with a 200-fold excess of the IC₅₀ concentration of each inhibitor, pitavastatin was able to induce expression of the LDL receptor mRNA most effectively. These results indicate that the effect of HMG-CoA reductase inhibitors on the upregulation of mRNA expression for reductase and LDL receptor differs for each and among other lipophilic inhibitors. NK-104 is most effective in inducing the expression of LDL receptor mRNA in Hep G2 cells (12).

In this experiment, we examined the pleiotropic effects of pitavastatin and atorvastatin in human umbilical vein endothelial cells using DNA chip technology to clarify the effect of statins on endothelial cells.

Materials and Methods

Cells and cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from a commercial source (Clonetics, San Diego, CA, Lot No.9F1034) and were grown in endothelial cell growth factor-containing medium-2 (EGM2, Clonetics) with 2% fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere. All experiments were performed in 4 passages. The cells were plated at a density of 3×10^5 per 100 mm dish and incubated 48 hours in the same medium with 1.1 μ M of pitavastatin and 6.6 μ M of atorvastatin. Pitavastatin and atorvastatin were dissolved in dimethylsulfoxide (DMSO, SIGMA, St. Louis, MO). The control cells were cultured in EGM-2 with 2% FBS containing 0.01% DMSO in the absence of statins. Eight or 24 hours later, the cells were harvested and the mRNA was recovered by the method of Chomczynski and Sacchi (13).

Microarray analysis of cRNA

One microgram of mRNA was used to generate first-strand cDNA by using a T7-linked oligo (dT) primer (Amersham Pharmacia Biotech, UK Ltd, Little Chalfont, England). After second-strand synthesis, *in vitro* transcription was performed with biotinylated UTP and CTP (Enzo Diagnostics Inc, New York, NY), resulting in a 40- to 80-fold linear amplification of RNA. Amplified cRNA was purified on an affinity resin column (Rneasy Mini Kit, Qiagen, Valencia, CA) and quantified by spectrophotometry. Forty micrograms of biotinylated RNA was fragmented into 50- to 150-nt units (14) before overnight hybridization to the oligonucleotide gene chip, the HuGene human FL array (Affymetrix, Santa Clara CA)(15,16).

These arrays contain probe sets for 6416 human genes (5223 known genes and 1193 expressed sequence tags). As the probe sets for certain genes were present more than once on the array, the total number of genes on the array was 7227. Fragmented cRNA and sonicated her-

ring sperm DNA, up to a final concentration of 0.1 mg/ml, were added to the hybridization buffer containing 1.0 M NaCl, 10 mM Tris-HCl (pH 7.6), and 0.01% Triton X-100 (ST-T). The hybridization mixture was heated to 99°C for 5 min and at 45°C for 5 min before injection of the sample into the probe array cartridge. Hybridizations were carried out at 45°C for 16 hours with mixing on a rotisserie at 60 rpm. Following hybridization, the solutions were removed and arrays were filled with Non-Stringent Wash Buffer (6X SSPE, 0.01% Tween-20, 0.05% Anifform). Hybridized arrays were stained with 5.0 μ g/ml of streptavidin/phycoerythrin (Molecular Probes, Eugene, OR) and 2.0 mg/ml of acetylated BSA (Sigma) in 1XST-T at 40°C for 15 min. After being washed, probe arrays were scanned two times at a resolution of 3 μ M using the GeneChip system confocal scanner made for Affymetrix by Hewlett-Packard. Intensity values were scaled such that the overall intensity for each chip of the same type was equivalent.

Quantitative analysis

The intensity of each feature in the array was captured with GENECHIP SOFTWARE (Affymetrix), and a single raw expression level for each gene was derived from the 20 probe pairs representing each gene by means of a trimmed mean algorithm. A threshold of 20 units was assigned to any gene with a calculated expression level below 20, because discrimination of expression below this level could be determined with confidence. Results were represented by two values, average difference and fold change (17). The average difference (Avg. Diff.) was calculated by taking the difference between the perfect match (PM) and mismatch (MM) of every probe pair and averaging the differences over the entire probe set:

$$\text{Avg. Diff.} = \Sigma (\text{PM} - \text{MM}) / (\text{pairs In Avg.})$$

The Avg. Diff. of a transcript was directly related to its expression level, an estimate of the fold change of the transcript between the control and experimental samples could be calculated. First, the Normalized or Scaled Avg. Diff. Values were recomputed with both the experimental and control data sets to include only probe pairs that were used in both the control and experimental arrays. Next, the Avg. Diff. Change was determined as follows:

$$\text{Avg. Diff. Change} = \text{Avg. Diff.}_{\text{exp}} - \text{Avg. Diff.}_{\text{control}}$$

Then, the fold change (FC) was calculated with this equation:

$$\text{FC} = \left(\frac{\text{Avg. Diff. Change}}{\max [\min (\text{Avg. Diff.}_{\text{exp}}, \text{Avg. Diff.}_{\text{control}}), Q_M * Q_C]} \right) + \begin{cases} +1 & \text{if Avg. Diff.}_{\text{exp}} \geq \text{Avg. Diff.}_{\text{control}} \\ -1 & \text{if Avg. Diff.}_{\text{exp}} < \text{Avg. Diff.}_{\text{control}} \end{cases}$$

Where $Q_c = \max(Q_{exp}, Q_{control})$
 And the Q multiplier $Q_M = 2.1$ in $50 \mu\text{m}$ feature arrays;
 2.8 in $24 \mu\text{m}$ feature arrays.

This equation permitted expression of the fold change as a positive number when the transcript had increased over its control level and as a negative number when the transcript level declined. If the Noise ($Q \cdot Q_M$) of either array was greater than the Avg. Diff. of the transcript (in either the control or experimental data), the fold change was calculated using the Noise.

Results

Fig. 1 shows the increase in mRNA levels for the low-density lipoprotein (LDL) receptor and for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in HUVEC after statin treatment. After 8 hours of treatment, both pitavastatin and atorvastatin increased the expression of the mRNA for these two genes by more than 3.5 fold over control levels. mRNA levels returned to control levels 24 hours after treatment. The effect on the two key genes in cholesterol metabolism, the LDL receptor and HMG-CoA reductase genes was prominent only at 8

Fig. 1. The average difference of HMG-CoA reductase (A) and LDL receptor (B) treated with statins in HUVEC. After HUVEC were treated with $1.1 \mu\text{M}$ of pitavastatin and $6.6 \mu\text{M}$ of atorvastatin for 8 and 24 hours, total RNA was prepared. The total RNA was analyzed as described in Materials and Methods.

hours post-treatment. These results indicate that both pitavastatin and atorvastatin inhibit cholesterol biosynthesis and that the feedback regulatory pathway functions well in HUVEC at the concentrations used in this study.

The suppression of mRNA for interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) by pitavastatin and atorvastatin is shown in Fig. 2. The mRNA levels for IL-8 are shown in Fig. 2a. Eight hours after treatment with pitavastatin and atorvastatin, IL-8 and MCP-1

Fig. 2. The average difference of interleukin-8 (A), monocyte chemoattractant protein-1 (B) and interleukin-13 (C) treated with statins in HUVEC. After HUVEC were treated with $1.1 \mu\text{M}$ of pitavastatin and $6.6 \mu\text{M}$ of atorvastatin for 8 and 24 hours, total RNA was prepared. The total RNA was analyzed as described in Materials and Methods.

mRNA levels were -3.2 and -4.1, respectively, of control levels. This suppressive effect continued for 24 hours. MCP-1 mRNA levels are shown in Fig. 1b. The difference between the treated and control cells was greater for MCP-1 than for IL-8. At 8 hours, the average difference for control, atorvastatin-treated and pitavastatin-treated cells was 238.2, 66.5, and 37.9 respectively. Treatment of HUVEC with statins for 24 hours, resulted in a decrease in MCP-1 mRNA levels. Both statins effectively suppressed the expression of these chemokine mRNAs at 8 hours and 24 hours after treatment. IL-13 is also known as a chemokine, but mRNA levels for this control cytokine, did not change markedly. This result indicates that statins are effective in suppressing pro-inflammatory gene expression in HUVEC.

Fig. 3 shows the effect of pitavastatin and atorvastatin treatment on mRNA levels for nitric oxide synthase (eNOS) and endothelin-1 (ET-1). Both statins induced eNOS mRNA expression and suppressed ET-1 mRNA expression at 8 and 24 hours after treatment. These results indicate that the two statins function in vasodilatation in HUVEC.

Fig. 4 shows the effect of pitavastatin and atorvastatin on plasminogen activator inhibitor-1 (PAI-1) and throm-

bomodulin (TM) mRNA levels. Although both statins suppressed PAI-1 mRNA levels by 75% at 8 hours post-treatment, their effect was less prominent after 24 hours. In contrast, both statins induced a 17-fold increase in TM mRNA levels 8 and 24 hours post-treatment. This result indicates that both statins suppress the expression of procoagulation genes and induce anti-coagulation activity in HUVEC.

Discussion

Large-scale clinical trials have demonstrated significant reductions in cardiovascular events following statin therapy. The observed benefit of statin therapy, however, may be greater in these trials than might be expected on the basis of lower lipid levels alone. In the present study, we studied the effect of statins on RNA expression profiles by DNA microarray analysis. Statins are known to induce the expression of mRNA for cholesterol biosynthetic enzymes and for the LDL receptor. In HUVEC, mRNA levels for HMG-CoA reductase and the LDL receptor increased 8 hours after the addition of either pitavastatin or atorvastatin, and returned to control levels at 24 hours post-treatment.

Fig. 3. The average difference of nitric oxide synthase-3 (A) and endothelin-1 (B) treated with statins in HUVEC. After HUVEC were treated with 1.1 μ M of pitavastatin and 6.6 μ M of atorvastatin for 8 and 24 hours, total RNA was prepared. The total RNA was analyzed as described in Materials and Methods.

Fig. 4. The average difference of plasminogen activator inhibitor-1 (A) and thrombomodulin (B) treated with statins in HUVEC. After HUVEC were treated with 1.1 μ M of pitavastatin and 6.6 μ M of atorvastatin for 8 and 24 hours, total RNA was prepared. The total RNA was analyzed as described in Materials and Methods.

We determined the expression levels of genes regulating endothelial functions, including chemotaxis, vascular constriction and dilatation, as well as blood coagulation. In HUVEC, IL-8 and MCP-1 mRNA expression was suppressed after statin treatment. The levels of ET-1 and PAI-1 mRNA were also reduced, while the levels of eNOS and thrombomodulin mRNA were increased. All of these effects seem beneficial in preventing atherosclerotic disorders. We found that, in contrast to HMG-CoA reductase and LDL receptor mRNA, these pleiotropic effects remain for up to 24 hours post-treatment. Especially in the case of the suppression of chemokine mRNA expression, the effect was more prominent at 24 hours than at 8 hours. The upregulation of HMG-CoA reductase and LDL receptor mRNA expression is mediated by SREBPs. The difference in time course may suggest the involvement of other transcriptional mechanisms regulated by the pleiotropic effect of statins.

IL-8 mediates the recruitment of neutrophils, while MCP-1 mediates the recruitment of monocytes. Pitavastatin suppressed the expression of mRNAs to a greater extent than did atorvastatin. In subjects with hypercholesterolemia, there was a four- and three-fold increase in the amount of IL-8 transcripts in peripheral blood mononuclear cells from patients, in unstimulated and LPS stimulated cultures, respectively (18). The serum level of IL-8 was higher in patients than normal subjects (19). Previous studies reported that administration of simvastatin to patients with coronary heart disorders significantly lowered plasma levels of MCP-1. The suppression of IL-8 and MCP-1 mRNA expression in endothelial cells by statins indicates a novel mechanism by which statins can prevent cardiovascular events.

Consistent with our results, a decrease in ET-1 mRNA and protein levels and upregulation of eNOS mRNA and protein expression in bovine aortic endothelial cells by statins have been reported by Hernandez-Perera *et al.* (4). Statins might influence vascular tone by modulating the expression of endothelial vasoactive factors.

The suppression of PAI-1 mRNA expression and the induction of thrombomodulin mRNA expression in endothelial cells might enhance the anti-coagulative activity. A decrease in plasma PAI-1 levels caused by statin treatment in hyperlipemic patients has been reported by Dangas *et al.* (20). Seeger *et al.* reported that an estrogen-statin combination inhibited the upregulation of PAI-1 mRNA expression in cultured endothelial cells (21). Thrombomodulin is an important anti-coagulation molecule on the surface of endothelial cells.

The initial step in atherosclerosis is the rapid targeting of monocytes to the sites of inflammation and endothelial injury. Cholesterol loading was found to induce IL-8 and MCP-1 expression, suggesting an association with foam cell formation. Oxidized LDL induces the expression of tissue factors in macrophages that also provide sites for

the polymerization of fibrin and decrease the anticoagulant activity of the endothelium by interfering with the expression of thrombomodulin. Plasminogen activator inhibitor-1 inhibits the migration of smooth muscle cells and, in the presence of vitronectin, promotes the clearance of thrombin by LDL receptor-related proteins at sites of endothelial injury. Pitavastatin and atorvastatin enhance the gene expression of thrombomodulin and eNOS, which may prevent the development of atherosclerotic lesions induced by oxidized LDL. These statins suppress the expression of IL-8 and MCP-1, two important chemokines for monocytes and neutrophils, which may prevent the accumulation of leukocytes at the lesion. These results show that statins are clinically effective because of their ability to change the gene expression profile in endothelial cells, thereby preventing vascular events.

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References

- (1) Endo A: The discovery and development of HMG-CoA reductase inhibitors. *Journal of Lipid Research*, 33: 1569-1582, 1992
- (2) Brown MS and Goldstein JL: A receptor-mediated pathway for cholesterol homeostasis. *Science*, 232: 24-47, 1986
- (3) Bellosta S, Bernini F, and Ferri N: Direct vascular effects of HMG-CoA reductase inhibitors. *Atherosclerosis*, 137: S101-109, 1998
- (4) Hernandez-Perera O, Perez-Sala D, Navarro-Antolin J, Sanchez-Pascuala R, Hernandez G, Diaz C, and Lamas S: Effects of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, atorvastatin and simvastatin, on the expression of endothelin-1 and endothelial nitric oxide synthase in vascular endothelial cells. *J Clin Invest*, 101 (12): 2711-2719, 1998
- (5) Gerard O and Roger T: Simvastatin, an HMG-CoA reductase inhibitor, improves endothelial function within 1 month. *Circulation*, 95 (5): 1126-1131, 1997
- (6) Fassbender K, Simons M, and Bergmann C: Simvastatin strongly reduces levels of Alzheimer's disease beta-amyloid peptides Abeta 42 and Abeta 40 in vitro and in vivo. *Proc. Natl Acad Sci USA*, 98 (10): 5856-5861, 2001
- (7) Suzuki H, Aoki T, Tamaki T, Sato F, Kitahara M, and Saito Y: Hypolipidemic effect of NK-104, a potent HMG-CoA reductase inhibitor, in guinea pigs. *Atherosclerosis*, 146: 259-270, 1999
- (8) Aoki T, Nishimura H, Nakagawa S, Kojima J, Suzuki H, Tamaki T, Wada Y, Yokoo N, Sato F, Kimata H, Kitahara M, Toyoda K, Sakashita M, and Saito Y: Pharmacological profile of a novel synthetic inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A re-

- ductase. *Arzneimittel-Forschung Drug Research*, 47: 904-909, 1997
- (9) Yamada N, Goto Y, and Saito Y: Clinical effect of NK-104 (Nisvastatin) on hyperlipidemia -double-blind comparative dose-finding study in three parallel groups-. XIII International Symposium on Drugs Affecting Lipid Metabolism, Abstracts 129, 1998
 - (10) Ohnaka K, Shimoda S, Nawata H, Shimokawa H, Kaibuchi K, Iwamoto Y, and Takayanagi R: Pitavastatin enhanced BMP-2 and osteocalcin expression by inhibition of Rho-associated kinase in human osteoblasts. *Biochem Biophys Res Commun*, 287: 337-342, 2001
 - (11) Takemoto M, Kitahara M, Yokote K, Asaumi S, Take A, Saito Y, and Mori S: NK-104, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, reduces osteopontin expression by rat aortic smooth muscle cells. *Br J Pharmacol*, 133 (1): 83-88, 2001
 - (12) Morikawa S, Umetani M, Nakagawa S, Yamazaki H, Suganami H, Inoue K, Kitahara M, Hamakubo T, Kodama T, and Saito Y: Relative induction of mRNA for HMG CoA reductase and LDL receptor by five different HMG-CoA reductase inhibitors in cultured human cells. *J Atheroscler Thromb*, 7 (3): 138-144, 2000
 - (13) Chomczynski P and Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analy Biochem*, 162: 156-159, 1987
 - (14) Wodicka L, Dong H, Mittmann M, Ho MH, and Lockhart DJ: Genome-wide expression monitoring in *Saccharomyces cerevisiae*. *Nat Biotechnol*, 15: 1359-1367, 1997
 - (15) Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, and Brown EL: Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol*, 14: 1675-1680, 1996
 - (16) Chee M, Yang R, Hubbell E, Berno A, Huang XC, Stern D, Winkler J, Lockhart DJ, Morris MS, and Fodor SA: Accessing Genetic Information with High-Density DNA arrays. *Science*, 274: 610-614, 1991
 - (17) Ishii M, Hashimoto S, Tsutsumi S, Wada Y, Mastsushima K, Kodama T, and Aburatani H: Direct Comparison of GeneChip and SAGE on the quantitative accuracy in transcript profiling analysis. *Genomics*, 68: 6694-6704, 1999
 - (18) Porreca E, Sergi R, Baccante G, Reale M, Orsini L, Febbo CD, Caselli G, Cuccurullo F, and Bertini R: Peripheral blood mononuclear cell production of interleukin-8 and IL-8-dependent neutrophil function in hypercholesterolemic patients. *Atherosclerosis*, 146 (2): 345-350, 1999
 - (19) Zhou RH, Shi Q, Gao HQ, and Shen BJ: Changes in serum interleukin-8 and interleukin-12 levels in patients with ischemic heart disease in a chinese population. *J Atheroscler Thromb*, 8: 30-32, 2001
 - (20) Dangas G, Smith DA, Unger AH, Shao JH, Meraj P, Fier C, Cohen AM, Fallon JT, Badimon JJ, and Ambrose JA: Pravastatin: an antithrombotic effect independent of the cholesterol-lowering effect. *Thromb Haemost*, 83 (5): 688-692, 2000
 - (21) Seeger H, Wallwiener D, and Mueck AO: Lipid-independent effects of an estrogen-statin combination: inhibition of expression of adhesion molecules and plasminogen activator inhibitor-I in human endothelial cell cultures. *Climacteric*, 4 (3): 209-214, 2001