Fluvastatin Alters Platelet Aggregability in Patients With Hypercholesterolemia
Possible Improvement of Intraplatelet Redox Imbalance via HMG-CoA Reductase

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**Background**—Hypercholesterolemia enhances platelet aggregability. Statins have beneficial effects on cardiovascular events. The purpose of this study is to investigate whether statins inhibit platelet aggregation and, if so, the mechanisms.

**Methods and Results**—Twelve patients with hypercholesterolemia were prospectively randomized in a crossover design to receive either fluvastatin (20 mg/d) or colestimide (3000 mg/d) for 12 weeks. The subjects were switched to the opposite arm for additional 12 weeks. Before and after first and second treatments, experiments were performed. Eleven age-matched volunteers with normal lipid profiles served as controls. ADP-induced platelet aggregation, platelet-derived nitric oxide (PDNO) release, intraplatelet levels of GSH and GSSG, and intraplatelet nitrotyrosine production during platelet aggregation were measured. Fluvastatin and colestimide equally lowered total and low density lipoprotein cholesterol levels in hypercholesterolemia. Platelet aggregation was greater in hypercholesterolemia than in normocholesterolemia before treatment and was altered by fluvastatin. PDNO release, intraplatelet glutathione level, and GSH/GSSG ratio were lower in hypercholesterolemia than in normocholesterolemia before treatment and were increased by fluvastatin. Intraplatelet nitrotyrosine formation was greater in hypercholesterolemia than in normocholesterolemia, and decreased by fluvastatin. Colestimide did not have such effects. In vitro application of fluvastatin dose-dependently inhibited platelet aggregation. Furthermore, in vitro application of fluvastatin dose-dependently inhibited platelet nitrotyrosine expressions and the inhibitory effects by fluvastatin were reversed by preincubation with geranygeranylpyrophosphate.

**Conclusions**—Fluvastatin altered platelet aggregability in hypercholesterolemic patients in a cholesterol-lowering independent manner, which was partly mediated by the improvement of intraplatelet redox imbalance. (*Arterioscler Thromb Vasc Biol.* 2007;27:1471-1477.)

**Key Words:** lipids | platelets | aggregation | redox | statins

Hypercholesterolemia is one of the major risk factors for the development and progression of atherothrombosis. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors, statins, are cholesterol-lowering agents. Several large clinical trials have demonstrated that statins markedly decrease cardiovascular events in hypercholesterolemic subjects. In subgroup analysis and meta-analysis of those trials, the risk for cardiovascular events in patients treated with statins is significantly lower than the expected risk at the same levels of serum cholesterol (overlap phenomenon). Moreover, a clinical trial showed that, in patients with acute coronary syndromes, statins reduced ischemic events at the earlier stage after treatment, which is too short for the beneficial effects of statins on vascular morphological changes. These findings suggest the pleiotropic effects of statins.

Platelet aggregability is enhanced in hypercholesterolemic animals and patients. We have previously shown that hypercholesterolemia is associated with impairment of platelet-derived nitric oxide (PDNO) releases from human platelets, and that impairment of the platelet L-arginine–NO pathway plays an active role in arterial thrombus formation in an experimental thrombosis model. There is increasing evidence that statins alter platelet aggregability in hypercholesterolemic patients. However, previous studies were conducted without active controls. Thus it still remains unclear whether the antiplatelet effects of statins are attributable to the cholesterol lowering effect or pleiotropic effects. Accordingly, in humans we investigated in a crossover design whether fluvastatin altered platelet aggregability, independently of cholesterol levels by using anion-exchange resin as a control and, if
so, the mechanisms of fluvastatin-induced platelet inhibition by focusing PDNO and intraplatelet redox status.

Methods

Study Subjects
Crossover design of drug treatment was applied to patients with hypercholesterolemia (n = 12). After randomization and with open label method, patients were treated with either HMG-CoA reductase inhibitor, statin (Fluvastatin, 20 mg/d) or anion-exchange resin (Co-lestidime; 3000 mg/d) for 12 weeks, and then switched to the other drug for additional 12 weeks. There was no washout period between them. Blood was collected before treatment, after the first treatment, and after the second treatment, and then ex vivo experiments were performed. Age-matched 11 healthy volunteers with normal lipid profiles were served as controls. There were no significant differences in age, gender, body mass index, blood pressure, plasma glucose between hypercholesterolemic subjects and normocholesterolemic subjects (Table). All female subjects were postmenopausal. No patients had symptomatic coronary artery diseases or family history of premature cardiovascular diseases. All subjects were nonsmoker, nonobese, nondiabetic, and normotensive. No subjects were on medications such as antplatelet agents or vitamin supplements. Additional patients with hypercholesterolemia (n = 11) were enrolled to carry out the in vitro platelet study. The protocol was approved by the Institutional Ethic Committee. Written informed consent was obtained from all subjects.

Preparation of Washed Platelets
Platelet suspensions were prepared as described previously. Briefly, blood (20 mL) was collected by venipuncture into a plastic tube containing 3.15% trisodium citrate. Platelet-rich plasma and platelet-poor plasma were prepared according to the previously described method. The platelet counts were adjusted to 2 × 10^11 platelets/mL in Tyrode solution, composition of which was described previously.

Measurements of Platelet Aggregation
We measured ADP-induced platelet aggregations as described previously. In brief, ADP (1, 2, 5, and 10 μmol/L) were added to the washed platelet suspensions. Platelet aggregation was assessed when the maximum aggregation was obtained at 7 minutes after ADP stimulation. Light transmission was monitored by using platelet aggregometer (MDM Hematracer, MC Medical Co). The aggregation was calculated as an index of intraplatelet oxidative stress.

Measurements of Platelet Aggregation in Human Platelets
Proteins were extracted from washed platelets as described previously. In brief, ADP (1, 2, 5, and 10 μmol/L) were added to the washed platelet suspensions. Platelet aggregation was assessed when the maximum aggregation was obtained at 7 minutes after ADP stimulation. Light transmission was monitored by using platelet aggregometer (MDM Hematracer, MC Medical Co).

In platelet aggregation study in vitro, washed platelet suspensions were preincubated with the various concentrations of fluvastatin (0, 1, and 10 μmol/L) and fluvastatin (10 μmol/L) plus geranylgeranylpseudophosphate (1 μg/mL) for 1 hour, and then ADP (5 μmol/L) was added to the washed platelet suspensions.

Measurements of PDNO
We measured NO by using a selective NO meter (Model N0-501, Inter Medical Co) as described previously. After the baseline electric was stabilized, an ADP-induced (50 μmol/L) electrical current was recorded at the rate of 20 mm/min and a change in the peak electrical current was considered as an index of the NO release. It is known in human platelets that other agonists such as thrombin and thromboxane A2 analog (U46619) induce platelet-derived nitric oxide (PDNO) but to a lesser extent than ADP. Furthermore, collagen causes platelet adherence to the microelectrode and distortion of the signal. On the basis of these lines of evidence, we therefore chose ADP as an agonist of PDNO release and did not test platelet aggregation responses to other agonists in the present study.

Detection of Intraplatelet Nitrotyrosine
We measured intraplatelet nitrotyrosine production with a modified method of a previous study. Immunolabeling was performed by using a polyclonal antibody to nitrotyrosine as a primary antibody and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) as a secondary antibody, and then analyzed with the FACScan (Becton-Dickinson). The results were expressed as the percent changes in nitrotyrosine-specific staining of platelets by PDNO-induced platelet aggregation in each group.

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Measurements of Intraplatelet Redox Status
We measured intraplatelet glutathione (HPLC) and GSSG (the oxidized form of glutathione) by high-performance liquid chromatography (HPLC) with an electrochemical detection system (ECD-300, Eicom Co) as previously described. The analytical column was a 150 × 4.6-mm, SC-5 ODS (Eicom Co). For HPLC measurements, PRP and PPP were mixed with HClO4 (final concentration, 6%), sonicated for 5 seconds with a tip sonicator (Model MS-50, Heat Systems-Ultrasonics Inc), and centrifuged at 12 000g for 2 minutes. The supernatant was then stored at −80°C until injection into the HPLC column. Intraplatelet GSH/GSSG ratio was calculated as an index of intraplatelet oxidative stress.

Western Blot Analysis of HMG-CoA Reductase in Human Platelets
Proteins were extracted from washed platelets as described previously. Proteins (140 mg for lane 1 and 280 mg for lane 2) were separated by SDS-PAGE, and transferred onto a polyvinylidene difluoride membrane. The membrane was then treated with goat polyclonal antibody raised against human HMG-CoA reductase (Santa Cruz Biotechnology). The resultant immunocomplexes were visualized with an enhanced chemiluminescence system according to the manufacturer’s instructions (ECL, Amersham Bioscience).

Statistical Analysis
Values are presented as means ± SD. Statistical comparisons between groups were performed by an unpaired Student t test. Multiple comparisons were analyzed by two-way repeated-measures ANOVA with a post hoc Scheffé test. The relationship between two variables was analyzed by use of linear regression analysis. Differences were considered statistically significant at P < 0.05.

Results
Baseline Lipid Profiles of the Study Subjects
The levels of total cholesterol (P < 0.01) and low density lipoprotein (LDL)-cholesterol (P < 0.01) were significantly higher in hypercholesterolemia than in normocholesterolemia, whereas high density lipoprotein (HDL)-cholesterol...
and triglycerides were similar between the groups (supplemental data I, available online at http://atvb.ahajournals.org).

Effects of Treatments on Serum Lipid Profiles
Fluvastatin significantly lowered levels of total cholesterol ($P<0.01$) and LDL-cholesterol ($P<0.01$) to the levels of normocholesterolemic subjects (supplemental data I). Fluvastatin did not change HDL-cholesterol and triglyceride. The effects of colestimide on lipid profiles were similar to those of fluvastatin (supplemental data I).

Platelet Aggregation
Representative ADP-induced platelet aggregations were shown in a subject with hypercholesterolemia at baseline, after fluvastatin, and after colestimide (Figure 1A). And, pooled data are shown in Figure 1B. Platelet aggregation was significantly greater in hypercholesterolemia than in normocholesterolemia before treatment ($P<0.01$; Figure 1B). Platelet aggregation was significantly reduced after the treatment with fluvastatin ($P<0.01$) but not after treatment with colestimide (Figure 1B). Furthermore, platelet aggregation after treatment with fluvastatin was significantly smaller than that in normocholesterolemia ($P<0.01$; Figure 1B). In vitro application of fluvastatin dose-dependently inhibited platelet aggregations (Figure 2A).

HMG-CoA Reductase in Human Platelets
The expression of HMG-CoA reductase was confirmed in human platelets (Figure 2B).

PDNO Releases
The PDNO release was significantly lower in hypercholesterolemia than in normocholesterolemia ($P<0.01$; Figure 3). The PDNO release was significantly increased ($P<0.01$) after the treatment with fluvastatin but not after the treatment with colestimide (Figure 3). Furthermore, PDNO after the treatment with fluvastatin was significantly greater than that in normocholesterolemia ($P<0.01$; Figure 3). The PDNO release was negatively correlated with platelet aggregation ($r=−0.60$, $P<0.01$; data not shown).

Intraplatelet Nitrotyrosine Production
The intraplatelet nitrotyrosine level was higher in hypercholesterolemia than in normocholesterolemia (Figure 4A). The
intraplatelet nitrotyrosine level was significantly decreased ($P<0.01$) after treatment with fluvastatin but not after treatment with colestimide (Figure 4A). Furthermore, intraplatelet nitrotyrosine after treatment with fluvastatin was significantly smaller than that in normocholesterolemia ($P<0.01$; Figure 4A). The intraplatelet nitrotyrosine level was negatively correlated with the PDNO release ($r=-0.56$, $P<0.01$; data not shown). In vitro application of fluvastatin dose-dependently inhibited platelet nitrotyrosine expressions and the inhibitory effects by fluvastatin were reversed by preincubation with geranylgeranylpyrophosphate. (Figure 4B).

**Intraplatelet Redox Status**

Before treatment, both the intraplatelet GSH level and GSH/GSSG ratio were lower in hypercholesterolemia than in normocholesterolemia (Figure 5A). They were significantly increased ($P<0.01$) after treatment with fluvastatin but not after treatment with colestimide (Figure 5A). Furthermore, both the intraplatelet GSH level ($P<0.05$) and GSH/GSSG ratio ($P<0.01$) after treatment with fluvastatin were significantly greater than those in normocholesterolemia (Figure 5B). The intraplatelet GSH level was positively correlated with the intraplatelet GSH/GSSG ratio ($r=0.83$, $P<0.01$; Figure 5C). Furthermore, the intraplatelet GSH/GSSG ratio was negatively correlated with platelet aggregation ($r=-0.60$, $P<0.01$; Figure 5D) and with the intraplatelet nitrotyrosine level ($r=-0.59$, $P<0.01$), and positively correlated with the PDNO release ($r=0.71$, $P<0.01$; data not shown).

**Discussion**

In the present study, we found in humans that fluvastatin altered platelet aggregation in association with the greater PDNO release and less oxidative stress. Colestimide did not have such effects.

**Hypercholesterolemia and Enhanced Platelet Aggregability**

Platelet aggregation is enhanced in hypercholesterolemic patients.8 In this study, because risk factors other than cholesterol were similar between normocholesterolemic and hypercholesterolemic subjects, the enhanced platelet aggregation in hypercholesterolemic patients must have been attributable to hypercholesterolemia. There are many mechanisms considered for the enhanced platelet aggregation. One of them is impaired PDNO release from platelets attributable to increased oxidative stress. Platelets possess the L-arginine–NO pathway through constitutive NO synthase in humans.20,21 PDNO during platelet aggregation increases the intraplatelet level of cyclic guanosine 3',5'-monophosphate and then inhibits platelet aggregation.22 Thus, PDNO acts as a negative feedback mechanism to inhibit not only platelet aggregation but also recruitment after aggregation.16 Consistent with our previous reports, we found that PDNO was lower in hypercholesterolemic patients. To address the mechanism for the decreased PDNO, we measured the intraplatelet nitrotyrosine level as a “footprint” of peroxynitrite, reaction product of superoxide anion, and NO.17 In our previous studies using an inhibitor of NO synthase or an intracellular scavenger of superoxide anion, we confirmed in platelets that nitrotyrosine was really a “footprint” of peroxynitrite, resulting from the interaction between NO and superoxide anion.11,12 In this study, the intraplatelet nitrotyrosine levels were greater in hypercholesterolemic patients than in normocholesterolemic subjects, indicating the decreased PDNO caused by oxidative stress.

To further address this issue, we measured intraplatelet levels of GSH and GSSG (oxidized form of GSH) and obtained the ratio of GSH/GSSG as a marker of intraplatelet
oxidative stress. Intracellular GSH serves as a free radical scavenger. Moreover, GSH not only regulates the intracellular redox status but also modulates the action and metabolism of NO. Indeed, it has been shown in cultured endothelial cells that depletion of GSH decreases synthesis of NO and that reduced thiol enhances NO activity. In our previous study, we showed that the intraplatelet ratio of GSH/GSSG was a marker of oxidative stress. Consistent with the result of nitrotyrosine, the intraplatelet ratio of GSH/GSSG was smaller in hypercholesterolemic patients than in normocholesterolemic subjects. Taken together, our results indicate that oxidative stress impairs PDNO bioavailability, resulting in enhanced platelet aggregability in hypercholesterolemic patients.

Effects of Fluvastatin on Platelet Aggregability
In the present randomized crossover study, 3-month treatment with fluvastatin and colestimide lowered plasma LDL levels in a similar manner without affecting other lipid profiles. Nevertheless, only fluvastatin altered platelet aggregability. Thus the altered platelet aggregability may have been caused by cholesterol-independent effects, ie, pleiotropic effects. Although previous investigators reported altered platelet aggregability by statins, it was unknown because of lack of active control drugs whether the effects of statins were attributable to cholesterol-lowering effects or pleiotropic effects. Our report is the first one demonstrating the altered platelet aggregability attributable to pleiotropic effects of fluvastatin. In this study, the presence of HMG-CoA reductase was confirmed in human platelets. Furthermore, in vitro application of fluvastatin at 10 μmol/L inhibited platelet aggregation. The concentration of fluvastatin at 10 μmol/L is pharmacological. It is 10-fold higher than peak plasma concentration of fluvastatin achieved clinically. These findings with the presence of HMG-CoA reductase in platelets suggest the possible direct effects of fluvastatin on platelets. However, the exact mechanisms are unclear.

Mechanisms of Fluvastatin-Induced Alteration of Platelet Aggregability
To examine the mechanisms by which platelet aggregability was altered by treatment with fluvastatin, we measured PDNO and oxidative stress. Only fluvastatin but not colestimide increased PDNO and decreased oxidative stress (increased GSH, increased GSH/GSSG, and decreased nitrotyrosine). Again, these antioxidative stress effects were not related to cholesterol levels, indicating pleiotropic effects of fluvastatin. In support of the present findings, we recently reported in rats that statin upregulated eNOS mRNA, augmented PDNO release, and attenuated platelet activation. Taken together, the present findings indicate that fluvastatin improves the imbalance of intracellular redox state, resulting in improvements of bioactivity of PDNO, and that this improvement in PDNO bioactivity may be responsible for the decreased platelet aggregation observed following fluvastatin treatment.

Interestingly, treatment with fluvastatin altered platelet function to a level better than in normocholesterolemic subjects. From the basis of the present study, it is plausible that these findings may be attributable to pleiotropic effects of fluvastatin, which has antioxidative action. However, the exact mechanisms remained unknown, because it is not ethically feasible to give fluvastatin to normocholesterol subjects. In the present study, several mechanisms must be considered for the antiplatelet effects of fluvastatin. Statins have binary effects, cholesterol lowering effect, and inhibition of synthesis of geranylgeranylpyrophosphate. The inhibition of geranylgeranylation of the small GTP-binding proteins, Rho and Rac, by decreasing geranylgeranylpyrophosphate with statins would increase endothelial NOS expression and decreases the formation of the superoxide anion. In this study, to address this issue, we investigated whether in vitro application of fluvastatin inhibited platelet nitrotyrosine expressions and the inhibitory effects by fluvastatin were reversed by supplementation of...
geranylgeranylpyrophosphate. As shown in Figure 4, fluvastatin decreased nitrotyrosine levels, and the effects were reversed by supplementation of geranylgeranylpyrophosphate. Our findings suggest that fluvastatin may reduce the oxidative stress at least via inhibition of synthesis of geranylgeranylpyrophosphate.

In this study in vitro, 1 μmol/L of fluvastatin inhibited platelet nitrotyrosine formation to a similar extent as 10 μmol/L fluvastatin did, whereas only 10 μmol/L fluvastatin inhibited platelet aggregation, but 1 μmol/L had no effect. These observations suggest that the platelet aggregation is not necessarily related to oxidative stress. However, the exact mechanisms are unclear from the present study. The relevance of the platelet inhibitory dose of 10 μmol/L fluvastatin should be discussed with respect to serum levels achieved in patients. Peak plasma concentrations are approximately 53.1±10.4 nmol/L and 471.8±145.8 nmol/L after single oral administration with 10 and 40 mg of fluvastatin, respectively. In a study with 40 mg of fluvastatin orally once daily for 6 days, peak plasma concentration was approximately 1.01±0.21 μmol/L. Thus, the antiplatelet dose (10 μmol/L) of fluvastatin used in the present experiment in vitro was somewhat pharmacological. In this study, because we could not measure plasma concentration of fluvastatin in vivo, it is unclear whether the beneficial effects of fluvastatin treatment are mediated by a direct effect of the drug on platelets. The direct antioxidative effect of fluvastatin on platelet did not necessarily explain its beneficial pleiotropic actions on platelets in vivo. Therefore, it is suggested that the antiplatelet effects of fluvastatin observed in vivo have been mediated by an indirect effect mediated through endothelial function.

**Limitations**

It is well known that platelet aggregability is enhanced in patients with hypercholesterolemia. However, it seems likely that the enhanced platelet aggregability is attributable to increased oxidative stress in chronic hypercholesterolemic state but not to hypercholesterolemia, per se. This was well demonstrated by our findings showing that colestimide lowered cholesterol levels to control levels in a relatively short period but did not improve oxidative stress. It is unknown whether chronic administration of colestimide for much longer periods would eventually improve oxidative stress and platelet aggregability. Furthermore, there were no thrombotic events during the treatment periods with fluvastatin or colestimide. However, 3 months for each were too short to...
assess the events. Further studies are needed for much longer periods to investigate whether fluvastatin decreases thrombotic events compared with colesteamide. Furthermore, fluvastatin differs from other statins with respect to its enzymatic metabolism. Whereas fluvastatin is metabolized by CYP450 2C9, other statins use CYP450 3A4 (eg, simvastatin or atorvastatin). CYP2C9 not only is the source of an endothelial-derived hyperpolarizing factor (EDHF) with antiplatelet activity, epoxysceostrienoic acids, but also a potential source of reactive oxygen species. Alterations of both factors by fluvastatin could be involved in the observed intraplatelet redox effects. Further clarification of antiplatelet actions of fluvastatin could be involved in the observed intraplatelet redox effects. Further clarification of antiplatelet actions of fluvastatin and comparisons with other statins will be necessary in future studies. Finally, it is well known that statin affects platelet function by changing the cholesterol content of platelet membranes, which alters membrane fluidity. We did not examine this issue in the present study.

In conclusion, the present study, to the best of our knowledge, provides the first demonstration in humans that statins alter the enhanced platelet aggregability possibly through improvement of intraplatelet redox imbalance. Our findings may contribute to the understanding of pathophysiological link of the pleiotropism of statin to beneficial cardiovascular effects.

Disclosures

None.

References

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