Polymorphonuclear Leukocytes May Impair Endothelial Function

Results of Crossover Randomized Study of Lipid-Lowering Therapies

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Objectives—To examine whether polymorphonuclear leukocytes (PMNs) in hypercholesterolemia (HC) are activated to generate large amount of superoxide in vivo and hence impair endothelial function and, if so, whether statins, which possess anti-inflammatory properties, may restore PMN-mediated endothelial dysfunction.

Methods and Results—At baseline, subjects with HC showed impaired endothelial function (P<0.001), estimated by flow-mediated vasodilation of the brachial artery, and increased susceptibility of low-density lipoprotein (LDL) to oxidation (P<0.0001) compared with control subjects. PMNs obtained from HC produced greater amount of superoxide (P<0.0001), showed higher adhesiveness to cultured endothelial cells (HUVECs) (P<0.0001), and impaired endothelial nitric oxide synthase (eNOS) Ser1177 phosphorylation of HUVECs compared with controls (P<0.001). Crossover administration of fluvastatin or colestimide for 3 months lowered LDL to the same levels (P<0.001 for both). Endothelial function was restored (P<0.0001). LDL oxidation (P<0.0001) and superoxide release from PMNs (P<0.0001) were diminished only in fluvastatin not in colestimide arm. Fluvastatin attenuated PMN adhesion to HUVECs (P<0.0001) and restored eNOS Ser1177 phosphorylation of HUVECs (P<0.001).

Conclusion—Statins may improve endothelial function at least in part by inactivating neutrophils independently of LDL reduction. Our results raise a novel concept that polymorphonuclear leukocytes may attack endothelia and play a pivotal role in the pathogenesis of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2005;25:1262-1267.)

Key Words: atherosclerosis ■ hypercholesterolemia ■ inflammation ■ oxidative stress ■ polymorphonuclear leukocytes

Several lines of evidence indicate that inflammation plays a pivotal role in the process of atherosclerosis.1 Although local inflammation of vulnerable plaque is responsible for the development of acute coronary syndrome,2,3 systemic inflammatory process as estimated by highly sensitive C-reactive protein (hsCRP) is closely associated with later cardiovascular diseases remain to be elucidated, reactive oxygen species (ROS) play a pivotal role in inflammation-mediated vascular injury. Polymorphonuclear leukocytes (PMNs) are ubiquitous effector cells in numerous inflammatory conditions.5 Relevance of PMN function is usually thought in terms of host defense in bacterial infections.5 PMNs produce a large amount of ROS through the respiratory burst and cause lipid peroxidation.7 In addition, on stimulation, PMNs release cytokines that promote endothelial cell activation and hence cell-to-cell adhesion.8 Collectively, these findings suggest a possible role of PMN in the development of vascular diseases via impairment of endothelial function. Widespread activation of PMN was demonstrated in subjects with hypercholesterolemia.9,10

Three-hydroxyl-3-methylglutanyl coenzyme A reductase inhibitors (statins) significantly decrease cardiovascular mortality associated with hypercholesterolemia.11 In vitro studies have shown that statins exert beneficial effects on vascular diseases by inhibiting leukocyte rolling and adhesion12 and by stabilizing endothelial nitric oxide synthase (eNOS) posttranslationally.13 Several clinical trials revealed that statins decrease markers of inflammation/oxidative stress14 and restore endothelium-dependent vasodilation15,16 in the placebo-controlled design. However, whether the restorations of endothelial function by statin are attributable to LDL reduction17 or to its pleiotropic effects remain to be tested by the alternative active treatment arm.

Accordingly, we hypothesized that PMN in hypercholesterolemia are activated, leading to oxidative stress in vivo and hence impair endothelial function, and, if so, statins may restore the PMN-mediated endothelial dysfunction independently of low-density lipoprotein (LDL) reduction. To test these hypotheses, we measured PMN activity, oxidative stress, and endothelial function in subjects with hypercholes-
terolemia and their age-matched controls, and we conducted a crossover trial using 2 different classes of lipid-lowering agents, fluvastatin and colestimide. Moreover, to investigate the direct interrelation between PMNs and endothelia, cell adhesion and eNOS phosphorylation as a process of eNOS activation in cultured human umbilical endothelial cells (HUVECs) were investigated before and after ex vivo exposure of PMNs obtained from hypercholesterolemic subjects with or without fluvastatin treatment.

**Methods**

**Subjects**

Eleven hypercholesterolemic patients with both total serum cholesterol >240 mg/dL and LDL cholesterol >160 mg/dL and 8 age-matched healthy subjects as controls were enrolled (Table). There were no significant differences in age, blood pressure, plasma glucose, or body mass index between hypercholesterolemic subjects and normocholesterolemic controls (Table). All female subjects were postmenopausal. No patients had symptomatic coronary artery diseases or family history of premature cardiovascular diseases. All subjects were nonsmokers, nonobese, nondiabetic, and normotensive. No subjects were using medication or vitamin supplements. The reproducibility of measurement, ie, coefficient of variation (CV) has been <10%, and the expected difference between drug was estimated ~20%. Based on this statistical background, the number for >90% of statistical power is estimated as 10 to 12 in the crossover design.

**Study Protocol**

The protocol was explained and written informed consent was obtained from each subject. The study was approved by Ethical Committee for Human Study in our institution. The study was performed with subjects in a supine position and in an air-conditioned room at 22 to 23°C. Patients were randomized to have fluvastatin 20 mg or colestimide 3 grams daily in a crossover design. Before and after 12 weeks of each treatment, endothelial function and biochemical and hematologic parameters were evaluated. The design of this study was a crossover trial in which drugs were not blinded, whereas the measurements of endothelial function or assay were completely blinded.

**Measurements of Vascular Function**

Flow-mediated vasodilation of the brachial artery was measured by a previously described noninvasive technique to assess endothelial function. Briefly, after a 10-minute equilibration period, with the use of a 10-MHz linear-array transducer and the SSA-380A system (Toshiba), the brachial artery was longitudinally imaged 5 cm proximal to the antecubital crease, twice at baseline, and then from 1 to 15 minutes continuously after the release of 4.5-minute upper arm arterial occlusion at 250 mm Hg of pressure with a 12.5-cm-wide cuff. Photographic images of end-diastolic frames were obtained and analyzed by a single investigator blinded to the subjects and sequences. The arterial diameter was measured by a caliper at the single most equivalently imaged site with side-by-side presentation. Flow-mediated vasodilation, ie, endothelium-dependent vasodilation, was determined as the maximal percent diameter change of the post-occlusion arterial diameter measurement relative to the mean of the corresponding 2 baseline measurements. The inter-observer and intra-observer variations of 2 baseline measurements were 2.8% and 1.6%, respectively, in our laboratory. Blood flow velocity was measured by Doppler at baseline and immediately after the release of cuff occlusion. Arterial blood flow was determined as arterial cross-sectional area times mean Doppler velocity. The magnitude of reactive hyperemia was calculated as the maximum flow divided by the baseline flow. As an internal control, we measured changes in the brachial diameter, ie, endothelium-independent vasodilation, induced by sublingual nitroglycerin (300 mg; Myocrol Spray; Toa Eiyo Co) given 15 minutes after the measurement of flow-mediated vasodilation. Five minutes after nitroglycerin administration, the scan was performed to assess endothelium-independent vasodilation.

**Activity of PMNs**

Activity of PMNs was estimated by superoxide generation during respiratory burst. Briefly, PMNs were separated from platelets and mononuclear cells by Ficoll-Hypaque density-gradient centrifugation. Purified PMNs were activated with 2 μg/mL of phorbol myristate acetate for 20 minutes and superoxide dismutase-inhibitable cytochrome c reduction, as an index of maximal superoxide generation by NADPH oxidase, was measured by the calorimetric method as previously described. NADPH oxidase is responsible for the rapid generation of superoxide from PMNs, ie, respiratory burst. The activity of this enzyme is not only determined by the subunits such as gp91phox or p22phox but also determined by their assembly. Because it has been considered that “total NADPH oxidase activity” is assessed by phorbol ester-induced superoxide release, we adopted this method.

**LDL Oxidation**

As a marker of systemic oxidative stress, susceptibility of LDL to oxidation was assessed by LDL lag time as previously described. LDL (1.019 < d < 1.063 g/mL) was prepared from plasma by sequential ultracentrifugation. EDTA and salt were removed from LDL using prepacked columns filled with Bio-Gel P6 as desalting gel. CuSO4 was added to LDL and the 234-nm absorption was measured for a period of 5 hours. It is difficult to examine if changes in PMN function are directly linked to endothelial function in vivo because statin would improve both PMN function and endothelium function. To resolve this issue, we examined their link ex vivo: effects of patient-derived neutrophils on HUVECs and on eNOS activation of HUVECs before and after statin. With this approach, we were at least able to examine whether the improved PMN function after statin affects the link between PMN and endothelium.

**Cell Adhesiveness**

Binding assay was performed with 3×10⁸ of separated PMNs coincubated with HUVECs in 6-well plates for 60 minutes at 37°C. Nonadherent cells were removed by gentle washing with phosphate-buffered saline 3 times. Adherent cells were counted by microscopy in a blinded manner.

**eNOS Ser1177 Phosphorylation**

To investigate the interrelation between PMNs and endothelia more directly, we performed additional ex vivo experiments before and after fluvastatin treatment (n=8). As an index of eNOS activation, the phosphorylation of Ser1177 on eNOS was assessed by Western blot analysis when HUVECs were coincubated with or without PMNs. HUVECs, which were cultured in the 6-well dishes to be confluent, were coincubated with or without 3×10⁶ of PMNs for 60 minutes in 5% CO₂ at 37°C. Then cells were stimulated with the calcium ionophore, A23187, for 3 minutes at concentration of 1 μmol, and they were washed twice with ice-cold phosphate-buffered saline. Total cell lysates were prepared by scraping the cells in lysis buffer (62.5 mMol/L Tris-HCl, 2% SDS, 10% glycerol, 50 mMol DTT, 0.01% BPP). After centrifugation for 5 minutes, samples were loaded onto 7.5% to 15% polyacrylamide gel (Readygels J; Bio-Rad) and...
electrophoresed. Gels were transferred to a nitrocellulose membrane, then the reaction was blocked by incubation in Tris-buffered saline (10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl) containing 0.1% (v/v) Tween 20 and 5% (v/v) nonfat dry milk for 1 hour, followed by 2-hour incubation with anti–phospho-eNOS-Ser1177 (Cell Signaling Technology) or anti-eNOS (Santa Cruz Biotechnology). Bands were visualized with the appropriate anti-rabbit horseradish peroxidase-conjugated secondary antibody using enhanced chemiluminescence substrate (Amerham Pharmacia Biotech) and Kodak X-OMAT film. The relative band densities were quantified using National Institutes of Health Image 1.62. The phosphorylations of eNOS were calculated as the ratio of phosphorylated eNOS to eNOS.

Statistical Analysis

Results were expressed as mean±SEM, and a value of \( P<0.05 \) was considered to be statistically significant. Comparisons between normal controls and hypercholesterolemic subjects were analyzed by Student t test. The lipid profiles, vascular function, cell adhesiveness, LDL lag time, and PMN activity, before and after treatment with fluvastatin or colestimide, were analyzed by 2-way ANOVA for repeated measurements and, if appropriate, followed by Schaeffe post hoc analyses.

Results

Baseline Results: Hypercholesterolemia Versus Control

Lipids profiles at baseline, total cholesterol \( (P<0.001) \), and LDL cholesterol \( (P<0.001) \) were significantly higher in the hypercholesterolemic subjects than in normocholesterolemic controls, whereas high-density lipoprotein cholesterol and triglycerides were similar between the groups (Figure 1).

Vascular Function

Both flow-mediated vasodilation \( (P<0.001) \) and nitroglycerin-induced vasodilation \( (P<0.001) \) were impaired in hypercholesterolemic patients compared with normocholesterolemic controls (Figure 2). The ratio of flow-mediated vasodilation to nitroglycerin-induced vasodilation was lower in hypercholesterolemic patients, indicating that endothelial function was predominantly impaired \( (P<0.001) \); Figure 2). The baseline diameter of brachial artery \( (3.86±0.57 \text{ mm versus } 3.80±0.48 \text{ mm, not significant}) \) and hyperemic flow response

Cell Adhesiveness

The number of adherent PMNs to HUVECs after 60-minute exposure was significantly greater in hypercholesterolemic subjects \( (P<0.0001) \); Figure 3).

Superoxide Generation

Unless stimulation by phorbol myristate acetate, superoxide production of PMN was not detectable. We measured superoxide from PMNs stimulated with phorbol myristate acetate. The production of superoxide from PMNs was significantly greater in hypercholesterolemic subjects \( (P<0.0001) \); Figure 4).

LDL Oxidation

Susceptibility of LDL to oxidation assessed by LDL lag time was curtailed in hypercholesterolemic patients \( (P<0.0001) \); Figure 4).
Effects of Lipid-Lowering Therapies: A Crossover Trial

Lipids profiles after 12-week treatment of fluvastatin or colestimide and total and LDL cholesterol were significantly decreased to the similar levels (P<0.0001 for both by ANOVA; Figure 1). The reductions of total or LDL cholesterol were not different between fluvastatin and colestimide groups. High-density lipoprotein cholesterol and triglycerides levels were not altered.

Vascular Function

After treatment with fluvastatin, flow-mediated vasodilation was restored to the control levels (P<0.0001 by ANOVA; Figure 2). Nitroglycerin-induced vasodilation was also improved in fluvastatin-treated subjects (P<0.05 by ANOVA; Figure 2), although the magnitude was rather smaller than that of flow-mediated vasodilation. Thus, the ratio was greater after fluvastatin treatment, indicating that endothelial function was predominantly improved (P<0.001 by ANOVA; Figure 2). However, colestimide treatment had no effect on vascular functions despite its lipid-lowering effects (Figure 2). The baseline diameter of brachial artery (fluvastatin 3.73±0.42 versus colestimide 3.79±0.55 mm, not significant) and hyperemic responses (fluvastatin 292±74 versus colestimide 290±56%, not significant) were not altered by either treatment.

Superoxide Generation

After treatment with fluvastatin, superoxide production was significantly attenuated compared with pretreatment, whereas colestimide had no effect on superoxide production by PMNs (P<0.001 by ANOVA; Figure 4). Furthermore, there was a significant inverse correlation between superoxide production by PMNs and flow-mediated vasodilation (r=−0.49, P<0.03).

LDL Oxidation

After treatment with fluvastatin, the LDL lag time was significantly prolonged compared with pretreatment, whereas colestimide had no effect on the LDL lag time (P<0.001 by ANOVA, Figure 2). Furthermore, there was a significant positive correlation between the LDL lag-time and flow-mediated vasodilation (r=0.74, P<0.0002).

Order Effects

For ethical reasons, our study had no washout period. By 2-way ANOVA for repeated measurements, we found no order effects on endothelial function and PMN activity. In addition, by post hoc analyses, which compared the effects of the same lipid-lowering agents between the statin–resin group and the resin–statin group, there was no difference on drug effects irrespective of the order.

Ex Vivo Interrelation Between PMN and Endothelia: Cell Adhesiveness

The number of adherent PMNs obtained from fluvastatin-treated subjects to HUVECs was significantly less compared with pretreatment (P<0.0001 by ANOVA; Figure 3). Colestimide treatment did not influence the PMN–HUVEC adhesiveness.

eNOS Ser^1177 Phosphorylation

The exposure of PMNs obtained from hypercholesterolemic subjects impaired eNOS phosphorylation of HUVECs (control 0.88±0.08, hypercholesterolemia 0.66±0.05; P<0.001). Fluvastatin restored the phosphorylation of eNOS to the nearly normal levels (P<0.001; Figure 5).

Discussion

The salient findings of this study are presented herein. In subjects with hypercholesterolemia, endothelial function was impaired and LDL oxidation was increased compared with controls. PMNs obtained from subjects with hypercholesterolemia generated greater amounts of superoxide and augmented cell adhesiveness to cultured endothelial cells compared with controls. Despite administration of fluvastatin or colestimide that lowered LDL to the similar levels, endothelium-dependent vasodilation and PMN–HUVEC adhesive-
ness were restored only in fluvastatin-treated subjects. Flu-
vastatin diminished the susceptibility of LDL to oxidation and attenuated superoxide release from PMNs, whereas colestimide had no effects. The exposure of PMNs obtained from hypercholesterolemic subjects on HUVECs impaired eNOS Ser1177 phosphorylation of eNOS, whereas chronic fluvastatin treatment normalized eNOS Ser1177 phosphorylation. These findings suggest that activated inflammatory cells in hyper-
cholesterolemia may impair endothelial function through oxidative stress, and fluvastatin may improve endothelial function by its anti-inflammatory properties against inflam-
more inflammatory cells independently of LDL reduction.

Oxidative Stress in Hypercholesterolemia
Oxidative stress estimated by LDL lag time was significantly increased in hypercholesterolemic subjects compared with controls. Although a number of indices of oxidative stress are advocated, LDL lag time, an index of susceptibility of LDL oxidation, has been widely used as a gold standard of the assessment of oxidative stress in humans.21 There are several sources of ROS in vivo such as mitochondrion, xanthine oxidase, catecholamine, or arachidonic acid. Among them, NADPH oxidase in phagocytic leukocytes generates a large amount of superoxide on stimuli, ie, respiratory burst.22 Therefore, we measured superoxide production as an index of oxidative stress in vivo. Likewise, LDL lag time and superoxide productions from PMNs were greater in hypercholesterolemic subjects than in normal controls, suggesting that activated PMNs may, at least in part, play a role in increased oxidative stress.

Pleiotropic Effect Beyond LDL Reduction
Several studies have shown that treatments with statins restore endothelial function15,16 and decrease the magnitude of inflammation or oxidative stress14 in hypercholesterolemic subjects. The paradigm, “pleiotropic effects of statins beyond LDL reduction” has been advocated and accepted extensive-
ly.24 However, one has to be cautious to interpret these previous results because most of them were placebo-
controlled studies that lacked the active control arm. We previously demonstrated that acute removal of plasma LDL by LDL apheresis normalized endothelial function in subjects with familial hypercholesterolemia.17 Therefore, “pleiotropic effects of statins beyond LDL reduction” in the aforemen-
tioned studies may be associated with the LDL reduction itself. In the present crossover study, 3-month treatment with fluvastatin or colestimide lowered plasma LDL levels in a similar manner without affecting other lipid profiles. Never-
theless, only fluvastatin treatment showed significant im-
provement of endothelial function, attenuation of superoxide production by PMNs, and reduction of lipid peroxidation. These results may indicate that fluvastatin restored the cross-talk between neutrophils and endothelia independently of LDL reduction. It is possible that cholesterol and LDL levels in PMNs are altered in hypercholesterolemic subjects, resulting in activation of those cells, and that fluvastatin (but not colestimide) may also ameliorate PMN functions by ameliorating lipid levels in the cells.

Endothelium–Polymorphonuclear Leukocytes Interaction
In this study, we tried to link the forearm endothelial vasodilator functions measured under physiological condi-
tions with PMN functions measured under nonphysiological conditions. There was a close link between physiological flow-mediated vasodilation and phorbol ester–stimulated res-
piratory burst of PMNs as an index of PMN activity. At basal conditions, superoxide production estimated by cyto-
chrome c reduction, which is a gold standard for in vitro measurement for ROS release from leukocytes, was not detected, indicating that PMNs were not activated without stimuli, which is consistent with previous reports.20 Therefore, we estimated the potential activity of PMNs during respiratory burst. In ex vivo experiments we counted the adhered PMNs to HUVECs in unstimulated conditions, indicating that PMNs may activate endothelium even in the absence of stimuli, ie, physiological conditions. Improvement of the response to NO was closely related to diminished superoxide release from PMNs. Several mechanisms can be attributed to this interrelationship. Superoxide released from PMNs may directly impair eNOS activity, partially caused by oxidation of tetrahydrobiopterin, an essential cofactor of eNOS, which causes unstabilization of dimeric structure of eNOS, leading to superoxide release by eNOS per se. In addition, endothelium-derived NO can be inactivated by superoxide-forming peroxinitrate, which also impairs biological effects of NO. We have demonstrated the close links among endothelial function, superoxide production by PMNs, and the LDL lag time before and after fluvastatin treatment in vivo. These findings suggest that oxidative stress-mediated aggression of PMN on endothelia may be attenuated by fluvastatin treatment. To investigate the interrelation between PMNs and endothelia more directly, we performed additional ex vivo experiments. The exposure of PMNs obtained from hypercholesterolemic subjects on HUVECs increased the adhesiveness between them and impaired calcium-
ionophore–induced Ser1177 phosphorylation of eNOS in HUVECs, a molecular marker of eNOS activation, suggest-
ing that PMNs from hypercholesterolemia directly activate endothelia and impair the cellular signaling for NO produc-
tion; however, fluvastatin decreased PMN–HUVEC adhe-
siveness and augmented eNOS Ser1177 phosphorylation. These findings may suggest that fluvastatin treatment hampers the attack of PMN against endothelia in humans. The molecular mechanisms of the anti-PMN activity of fluvastatin are beyond the scope of this study. However, it has been demonstrated that statins inhibit superoxide anion formation produced by NADPH oxidase in neutrophils because of a blockade of isoprenoid synthesis in vitro.25 Therefore, the beneficial effects of statins in the prevention of cardiovascu-
lar events could be caused in part by the manipulation of the regulatory system of the mevalonate pathway in leukocytes unrelated to cholesterol biosynthesis.

Study Limitation
We demonstrated the close link between endothelial function and PMN activity in the former half of this study, which does not necessarily indicate the direct causal role of neutrophils in
endothelial dysfunction or effects of the specific treatment. To resolve this issue, we examined their link ex vivo: effects of patient-derived neutrophils on normal cultured endothelial cells and on their eNOS phosphorylation. With this approach, we demonstrated PMN obtained from hypercholesterolemic subjects: (1) increased adhesiveness, ie, endothelial activation, between cultured endothelial cell and PMNs; and (2) impaired the phosphorylation of eNOS, an index of eNOS activity. These ex vivo results may suggest the attack of PMNs against endothelial cells. The drawback of this study is that we failed to dissect PMN activation from endothelial activation in our patients with mild hypercholesterolemia. Increased PMN adhesion to the endothelium could be caused by endothelial activation with hypercholesterolemia alone or with activation of other blood cells, such as monocytes and platelets. Thus, the increased PMN adhesion to the endothelium does not necessarily indicate PMN activation in this study. To support our conclusion, we showed direct evidence for PMN activation and the positive correlation between endothelial dysfunction and PMN activation under physiological conditions. To investigate the precise role of neutrophils on endothelial dysfunction, further studies, such as leukapheresis on endothelial dysfunction, may be required. In the present study, we did not present the data of hsCRP, because our preliminary results in a small size of samples failed to demonstrate a significant relationship between the cholesterol levels and hsCRP. We estimated PMN activity by measuring superoxide dismutase-inhibitable cytochrome c reduction during phorbol ester stimulation. Although the maximal NADPH oxidase activity can be anticipated from this method, unstimulated or receptor-stimulated activity of PMNs needs to be determined by alternative methods. Hypochlorous acid generated by myeloperoxidase also plays a pivotal role in PMN-mediated tissue injury. Recent studies have shown that plasma myeloperoxidase is elevated in patients with acute coronary syndrome, and its levels are associated with their prognosis. Thus, the role of myeloperoxidase in PMN-mediated endothelial dysfunction remains to be tested in further studies.

Perspectives

Our results raise a novel concept that inflammatory cells may attack endothelia and play a pivotal role in the pathogenesis of atherosclerosis. Recent large-scale trials have shown that statins improve clinical outcome in acute coronary syndrome, in which widespread activation of PMN has been demonstrated. Furthermore, PMNs may be associated with postprandial endothelial dysfunction via inflammation/oxidative stress. Thus, PMNs may become a novel target as the therapeutic strategy for cardiovascular diseases.

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