Low-Level Endotoxin Induces Potent Inflammatory Activation of Human Blood Vessels
Inhibition by Statins

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Background—Low-level endotoxemia (ie, ≥50 pg/mL) in apparently healthy subjects was recently identified as a powerful, independent risk factor for atherosclerosis.

Methods and Results—We treated human saphenous veins (HSVs) with low levels of endotoxin. Release of the proinflammatory chemokines interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) was measured by ELISA. Superoxide was determined by using the fluorescent probe dihydroethidium (HE), and monocyte binding was assessed with calcein-labeled U-937 cells. Three- to 4-fold increases in MCP-1 and IL-8 release were observed at endotoxin concentrations of 100 pg/mL; these increases were inhibited by the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor atorvastatin. Studies in cultured endothelial cells suggest that the mechanism is related to inhibition of isoprenylation (ie, geranylgeranylation) rather than cholesterol formation. Endotoxin produced dose-dependent increases in HE fluorescence that were inhibited by the superoxide dismutase mimics Tiron and MnTBAP. Endotoxin potently induced U-937 cell binding to HSV; binding was inhibited by both Tiron and atorvastatin. Toll-like receptor-4 expression was detected in cultured HSV endothelial and smooth muscle cells and in intact HSV.

Conclusions—Clinically relevant levels of endotoxin, as reported in ambulatory populations, have profound inflammatory effects on intact HSV. Inhibition of endotoxin-induced vascular inflammation might contribute to the beneficial effects of statins in treating atherosclerosis. (Arterioscler Thromb Vasc Biol. 2003;23:1576-1582.)

Key Words: toll-like receptor-4 • superoxide • monocytes • saphenous veins • atorvastatin

Atherosclerosis of coronary arteries and coronary bypass grafts frequently occurs in patients without traditional cardiac risk factors.1 Inflammatory markers have been correlated with increased risk of cardiovascular events,2 suggesting that chronic inflammation might play a major role in the development of atherosclerosis and restenosis in many patients.3,4 The factors responsible for vascular inflammation in atherosclerosis, however, are largely unknown.

One potentially important proatherosclerotic factor is circulating endotoxin, a unique glycolipid that comprises most of the outer leaflet of the outer wall of Gram-negative bacteria (GNB).5 This complex molecule, found exclusively in GNB, consists of a highly variable carbohydrate portion and a unique lipid A region that is highly conserved across many GNB species.5 Epidemiologic evidence suggests that endotoxemia at levels of ≥50 pg/mL constitutes a strong risk factor for the development of atherosclerosis, particularly among smokers.6,7 Moreover, chronic infections conferred an increased risk of atherosclerosis even in low-risk subjects who lacked conventional vascular disease risk factors.6,7 Low levels of endotoxemia in apparently healthy subjects might result from chronic or recurrent infection associated with the breaching of epithelial barrier function (eg, periodontitis, smoker’s bronchitis, diverticulitis, etc). The circulating endotoxin activates inflammatory cells (ie, macrophages and neutrophils) by binding to CD14 through a process that is modulated by lipopolysaccharide-binding protein.8 CD14 has an intracellular domain and interacts with Toll-like receptor-4 (TLR-4) to initiate cellular signaling. Very recently, humans with the Asp299Gly TLR-4 polymorphism, which is associated with attenuated airway responsiveness to endotoxin, were found to have a decreased risk of development of atherosclerosis.9

Little is known about the effects of low levels of endotoxin on intact human blood vessels. Loppnow and Libby10 reported release of interleukin (IL)-6 by isolated human saphe-
nous vein (HSV) explants in response to 10 µg/mL endotoxin. However, concentrations of endotoxin in the microgram per milliliter range are even higher than those seen in sepsis. Furthermore, concentrations of endotoxin >100 ng/mL can produce cellular activation independent of CD14 and TLR-4, and thus might not accurately reflect how blood vessels might respond to clinically relevant levels of endotoxin. 8,11–13 Although several studies demonstrated responses to endotoxin in cultured human endothelial cells and smooth muscle cells,14–16 extrapolation of data from cultured vascular cells to intact blood vessels is confounded by many variables, including the phenotypic and functional changes that vascular cells undergo when growing in culture. Therefore, studies performed to date yield limited insight into the potential role of low-level endotoxin as a mediator of inflammation in human blood vessels.

Atherosclerosis is a serious problem that affects HSVs used in coronary artery bypass surgery, with a 41% occlusion rate 10 years after implantation;17 the occlusion has been linked to chronic infection.17 We therefore examined the effects of low-level endotoxin on the production of proinflammatory cytokines, superoxide production, and monocyte binding in HSV. Importantly, expression of TLR-4 was evaluated. We also investigated atorvastatin as a potential inhibitor of endotoxin-induced inflammatory activation of human blood vessels.

Methods

Unused HSVs harvested for coronary artery bypass surgery were obtained from the operating room according to a protocol approved by the University of Iowa Human Subjects Office. For experiments, HSVs were cut into 2- to 3-mm segments. Human coronary artery endothelial cells (HCAECs) were purchased from Clonetics (San Diego, Calif). Endothelial (HSVECs) and smooth muscle (HSVSMCs) cells were cultured from HSV. Escherichia coli K12 LCD25 endotoxin was purchased from List Biologicals. U-937 histiocytic human lymphoma cells were purchased from American Type Culture Collection (Manassas, Va). IL-8 and monocyte chemoattractant protein-1 (MCP-1) were measured by ELISA, as previously described.18 Vascular O2- was detected by confocal microscopy with 10 μmol/L dihydroethidium (HE) staining for 30 minutes and a krypton/argon laser, as previously described.19

To measure monocyte adhesion, HSVs were treated with endotoxin, washed to remove any traces of endotoxin, and then incubated for 1 hour with calcein-labeled U-937 cells. After being washed to remove nonadherent U-937 cells, HSVs were prepared for frozen sectioning and cut into serial 8-µm slices, which were examined for U-937 cell binding by confocal laser scanning microscopy or fluorescence microscopy. TLR-4 expression in HSVs was examined by reverse transcriptase–polymerase chain reaction (RT-PCR), Western blotting, and immunohistochemistry. RT-PCR analysis was performed in cultured HSVECs and HSVSMCs. Western blot analysis was performed as described previously,20 with use of a mouse monoclonal anti–TLR-4 antibody generated against the extracellular domain of human TLR-4 (IMG-320, Imgenex). For immunohistochemistry, HSVs were cryosectioned and fixed in ethanol. Endogenous peroxidase was quenched with H2O2; Nonspecific background staining was blocked with normal horse serum in phosphate-buffered saline. After being stained with anti–TLR-4 antibody and rinsed, TLR-4 was visualized with avidin/biotin, followed by development in diamobenzidine and counterstaining in 10% Harris hematoxylin. For additional methodological details, please see http://atvb.ahajournals.org.

Results

To test the effects of endotoxin on chemotactic cytokine production, HSVs were incubated with 0 to 10 ng/mL endotoxin for 24 hours, and the media were assayed for release of IL-8 or MCP-1 (Figure 1). Because of the variability between individual patients in absolute amounts of IL-8 and MCP-1 released per milligram of tissue, release is expressed as a percentage of basal (control) levels. Near-maximal increases in IL-8 release were observed with 0.1 ng/mL endotoxin, with an ñ4-fold increase compared with control (Figure 1A). This concentration of endotoxin is clinically relevant, in that plasma levels in this range were associated with development of atherosclerosis in humans.6 IL-8 release increased in a time-dependent manner, with no detectable increase at 2 hours and a minimal response at 6 hours (data not shown). Endotoxin also produced dose-dependent increases in MCP-1 release, with an ñ8-fold increase observed in response to 10 ng/mL endotoxin (Figure 1B). Similar results were obtained in arterial tissue from 2 patients. (For details, please see http://atvb.ahajournals.org.)

Endotoxin is known to cause release of other cytokines, such as tumor necrosis factor-α (TNF-α). To rule out the possibility that the responses we observed might be occurring indirectly through secondary TNF-α release, we incubated HSVs with a blocking antibody to TNF-α (0.05 ng/mL MAB-610, R&D Systems) and tested whether this antibody
O₂ or MnTBAP, confirming that the signal originated from individual patients.

A total of 5 experiments with multiple sections of HSVs obtained as follows: LPS, 678% of control; LPS/Tiron, 101% of control; LPS/mevalonate (M). Binding of U-937 cells was determined, averaged for each individual experiment, and expressed as a percentage of control. Numbers of individual HSVs (from different patients) are indicated in the columns. *P<0.05 compared with 10 ng/mL LPS. 

The HE fluorescence was 295% of control at 1 ng/mL LPS and 422% of control at 10 ng/mL LPS with 0 (control), 1 or 10 ng/mL LPS, or 10 ng/mL LPS+10 μmol/L AT. Tissues were washed, incubated for 1 hour with calcein-labeled U-937 cells and 10 μmol/L HE to counterstain the vessel wall, and then examined by confocal laser scanning microscopy. B, Quantification of U-937 cell binding as a percentage of control. HSVs were preincubated for 24 hours without (C) or with (LPS, 0.1 to 10 ng/mL) endotoxin, with 10 ng/mL LPS+10 μmol/L AT, or with AT+100 μmol/L mevalonate (M). Binding of U-937 cells was determined, averaged for each individual experiment, and expressed as a percentage of control. Numbers of individual HSVs (from different patients) are indicated in the columns. *P<0.05 compared with control; #P<0.05 compared with 10 ng/mL LPS. C, HSVs were treated with vehicle or with 10 ng/mL LPS in the absence or presence of AT (doses and abbreviations are the same as for B). After 24 hours, the media (n=6 to 14) were assayed for IL-8 by ELISA and expressed as described in Figure 1.

Next, we investigated the effects of endotoxin on superoxide production by HSVs. Tissues were incubated for 4 hours with 0 (control), 1, or 10 ng/mL endotoxin, stained with HE, and then imaged with a laser scanning confocal microscope. Dose-dependent increases in HE fluorescence were observed in HSVs (Figure 2). The increases in HE fluorescence were observed throughout the vessel wall, suggesting enhanced O₂⁻ production in the intima, media, and adventitia. The HE fluorescence was blocked when the vessels were incubated with endotoxin in the presence of the O₂⁻ scavengers Tiron or MnTBAP, confirming that the signal originated from O₂⁻. These findings are consistent with a previous report in which high levels of endotoxin were observed to increase lucigenin-enhanced chemiluminescence in rat aorta.21

Adherence of monocytes to the endothelial lining of blood vessels is an early event in the atherogenic process, plays a role in plaque instability,22 and might involve IL-8, MCP-1, and O₂⁻. We therefore examined the adhesion of U-937 cells to endotoxin-treated HSVs. The first 3 panels of Figure 3A demonstrate that endotoxin caused marked increases in adherence of calcein-labeled U-937 cells (yellow-green) to the endothelial surface of the HSV (red). This potent induction of U-937 cell binding to HSV by endotoxin could have important implications for bypass graft disease in humans. Similar results were obtained with lipid A. (For details, please see http://atvb.ahajournals.org.) Statins have been reported to have important anti-inflammatory properties, in addition to their lipid-lowering effects. To determine whether endotoxin-induced U-937 cell binding could be inhibited by a statin, HSVs were incubated for 24 hours in RPMI containing 1% fetal bovine serum and 0 or 10 ng/mL endotoxin in the absence or presence of 10 μmol/L atorvastatin. Atorvastatin abolished the U-937 adherence resulting from treatment with endotoxin (Figure 3A, fourth panel). Similar results were obtained in 1 experiment with lovastatin (not shown). Figure 3B shows a summary of results from multiple experiments that examined U-937 binding to HSVs after treatment with 0.1 to 10 ng/mL endotoxin in the absence or presence of atorvastatin. In some experiments, 100 μmol/L mevalonate was included in the incubation to examine the mechanism of inhibition by atorvastatin. The effects of endotoxin on U-937 cell binding were concentration dependent, with maximal responses observed at 1 ng/mL (≈3.5-fold increase over control). Atorvastatin...
eliminated the induction of U-937 cell binding in response to 10 ng/mL endotoxin, an effect that was prevented by including mevalonate in the incubation medium. Atorvastatin also inhibited endotoxin-induced increases in IL-8 release, as shown in Figure 3C. Furthermore, in HSVs from 3 patients, atorvastatin (10 μmol/L) caused a 77±10% inhibition of MCP-1 release induced by lipopolysaccharide (10 ng/mL), which was reversed to 13±37% inhibition by coincubation with 100 μmol/L mevalonate (n=3; data not shown). These results suggest that atorvastatin inhibits the proinflammatory effects of endotoxin by preventing production of mevalonate, the immediate product of 3-hydroxy-3-methylglutaryl coenzyme A reductase, rather than through a nonspecific effect.

To further explore the mechanism of statin inhibition of endotoxin signaling, we performed a series of experiments with human HCAECs, which were recently reported to respond to nanogram levels of endotoxin. Treatment with endotoxin produced no measurable increase in IL-8 before 4 hours, with IL-8 levels increasing in a linear manner through 24 hours (Figure 4A), consistent with a mechanism that involves transcriptional activation and subsequent protein synthesis. Figure 4B shows that even the lowest lovastatin concentration tested, 0.1 μmol/L, produced a significant degree of inhibition. The degree of inhibition increased with increasing lovastatin concentration, with 50% inhibition occurring at ≈1 μmol/L. No effect on cell viability was observed at lovastatin concentrations <10 μmol/L.

Figure 4C shows that lovastatin inhibition of lipopolysaccharide signaling is reversed by mevalonate but not by the common sterol precursor squalene. Additionally, the effect of lovastatin is reversed by both geranylgeranyl pyrophosphate and geranylgeraniol. Because these compounds act at a step downstream of farnesylation, these results suggest that the statin inhibition of endotoxin signaling is due to inhibition of geranylgeranylation, rather than farnesylation. This conclusion is supported by the findings shown in the last 2 bars of Figure 4C. Using specific inhibitors of geranylgeranyl transferase and farnesyl transferase, we found that farnesylation transferase inhibitor had no effect on endotoxin signaling; however, blocking geranylgeranylation with geranylgeranyl transferase inhibitor also blocked endotoxin signaling.

Reactive oxygen species, such as O₂⁻, have been reported to increase production of chemotactic cytokines and stimulate invasion of monocytes into the vascular wall. Therefore, we examined whether endotoxin-induced U-937 cell binding could be inhibited by O₂⁻ scavengers. Tiron (10 mmol/L) substantially decreased endotoxin-induced monocyte adhesion to HSV segments (Figure 5). Likewise, MnTBAP reduced endotoxin-induced U-937 cell adhesion (not shown). These results suggest that O₂⁻ is an important mediator of endotoxin-induced inflammatory activation of HSVs.

The key lipid A recognition protein in the endotoxin receptor complex is thought to be TLR-4. Therefore, we performed RT-PCR to examine the expression of TLR-4 mRNA. Endothelial and smooth muscle cells were isolated from HSVs and grown to confluence in multwell plates for these experiments. Figure 6A shows that TLR-4 mRNA was detected in both types of cells. TLR-4 protein was detected in HSVs from 2 patients, as indicated by Western blotting (Figure 6B). This was further confirmed by immunohistochemical analysis performed on an HSV segment from a third patient (Figure 6C) and in isolated smooth muscle cells cultured from the HSV of a fourth patient (data not shown). The pattern of staining suggests that TLR-4 protein is present throughout the vessel wall, with strong staining along the endothelial surface and in the media. These results suggest that TLR-4 protein is expressed by resident cells in HSV.

Figure 4. Role of isoprenylation in endotoxin (lipopolysaccharide; LPS)-induced IL-8 release by HCAECs. A, Time dependence of LPS-induced IL-8 release. Confluent monolayers of HCAECs in 48-well plates were incubated for 0 to 24 hours with minimal essential medium (MEM) containing 2% fetal bovine serum±10 ng/mL LPS. At the indicated times, media were removed from sets of 4 wells and frozen for subsequent analysis for IL-8 by ELISA. Data represent mean±SEM of media collected from 4 individual wells for each condition. C, Inhibition of LPS-induced IL-8 release by lovastatin: concentration dependence. HCAECs were incubated for 24 hours as described in A with 10 ng/mL LPS, along with increasing concentrations (0.1 to 10 μmol/L) ofLovastatin. At the end of the incubation period, the media were collected and analyzed by ELISA for IL-8. Data represent mean±SEM of media collected from 4 individual wells for each condition in 1 of 2 experiments with similar results. C, Effect of isoprenylation modulators on LPS-induced IL-8 release by HCAECs. HCAECs were incubated for 24 hours with MEM containing 2% fetal bovine serum with no additives (Control), 10 ng/mL LPS (LPS), LPS±3 μmol/LLovastatin (Lov), or LPS±lovastatin±200 μmol/L mevalonate (Mev), 10 μmol/L squalene, 1 μmol/L geranylgeranyl pyrophosphate (GGPP), or 100 μmol/L geranylgeraniol (GG’ol). Some groups of wells were incubated with 10 ng/mL LPS±the farnesyl transferase inhibitor FTI-286 (FTI, 0.5 μmol/L) or the geranylgeranyltransferase inhibitor GGTI-277 (GGTI, 5 μmol/L). At the end of the incubation period, the media were collected and analyzed by ELISA for IL-8. Data represent mean values ±SEM expressed as a percentage of control of media collected from 4 individual wells for each condition in 1 of 3 experiments with similar results. *P<0.05 vs control.
Discussion

The hypothesis that circulating endotoxin participates in the pathogenesis of atherosclerosis is suggested by recent epidemiologic studies and by a study in an animal model of atherosclerosis. Endotoxin potently activates human macrophages, which play an important role in the development of atherosclerosis in humans. Our findings suggest that clinically relevant levels of endotoxin, as reported in ambulatory populations, have profound inflammatory effects on intact human blood vessels. The effects that we have observed (eg, induction of IL-8, MCP-1, superoxide, and U-937 binding) are potentially relevant to mechanisms of atherosclerosis in native vessels and bypass grafts. In addition, we have shown that atorvastatin inhibits endotoxin-induced U-937 cell binding to HSV, suggesting a potentially important non-cholesterol-lowering effect of statins in ameliorating atherosclerosis. Finally, we have demonstrated the expression of the endotoxin signaling receptor TLR-4 in HSV.

Chronic infection, with production of inflammatory cytokines, is thought to accelerate atherosclerosis. Two proinflammatory cytokines believed to play an important role in the initiation and progression of atherosclerosis are IL-8 and MCP-1. IL-8 was first identified and characterized as a neutrophil-activating protein produced by monocytes in response to very low levels of endotoxin. IL-8 is chemotactic for neutrophils and for T lymphocytes; moreover, it was recently shown to induce chemotaxis of freshly isolated peripheral blood monocytes and to promote their adhesion onto endothelial monolayers. MCP-1 is highly expressed in human atherosclerotic plaques and is thought to be a key factor in monocyte recruitment into subendothelial lesions.

Both IL-8 and MCP-1 have been shown to contribute to the pathogenesis of atherosclerosis in mouse models of hyperlipidemia. We report that low levels of endotoxin stimulate release of both IL-8 and MCP-1 from HSV and aorta. The endotoxin-induced release of IL-8 occurs independently of generation of TNF-α; however, whether other cytokines, such as IL-1, facilitate the generation of secondary cytokines and/or reactive oxygen species remains to be determined. Furthermore, as little as 0.1 ng/mL endotoxin increases adhesion of U-937 cells to HSV. Synthetic lipid A also potently induced U-937 cell binding to HSV. These findings suggest that the subnanogram levels of endotoxin found in apparently healthy subjects in the Bruneck study are sufficient to induce cytokine release and inflammatory cell binding in intact human blood vessels.

There is a growing awareness that statins might have important anti-inflammatory effects, in addition to their lipid-lowering effects. For example, cerivastatin inhibited firm adhesion of U-937 cells to IL-1β-activated human umbilical vein endothelial cells while downregulating surface expression of CD11a, CD18, and VLA4 and inhibiting actin polymerization in the U-937 cells. Our findings show that atorvastatin blocked endotoxin-induced U-937 adhesion to HSV. The mechanism for this inhibition of monocyte binding
by atorvastatin appears to be reduced synthesis of mevalonate. Reduction in mevalonate formation could interrupt endotoxin signaling at a number of steps, leading to inhibition or downregulation of chemoattractant molecules such as intercellular adhesion molecule-1 and vascular cell adhesion molecule; cytokines such as MCP-1 or IL-8; and/or production of reactive oxygen species. Our studies performed in cultured HCAECs further suggest that inhibition of isoprenoid formation, rather than cholesterol, is responsible for the effects of statins on endotoxin-induced inflammatory activation. Moreover, pharmacologic studies suggest that statins block endotoxin signaling by inhibiting geranylgeranylation, rather than farnesylation. The specific signaling molecules in the endotoxin pathway that are potential targets for geranylgeranylation remain to be determined.

Superoxide levels are elevated in atherosclerotic blood vessels and are thought to play an important role in the pathogenesis of the disease. Early studies with chemiluminescence techniques suggested that the increased superoxide in atherosclerotic blood vessels was localized to the endothelium. Recent studies with confocal microscopy with HE, however, indicate that the superoxide is distributed throughout the vascular wall in atherosclerosis. A similar pattern of distribution of superoxide was observed in the present study after treatment of HSV with endotoxin. Thus, our findings suggest that circulating endotoxin could potentially contribute to the increased vascular superoxide production observed in human atherosclerosis.

Superoxide and other reactive oxygen species are thought to play a key role in the pathogenesis of atherosclerosis by mechanisms such as scavenging nitric oxide and stimulating the formation of oxidized LDL. In addition, previous studies have shown that oxidative stress increases production of MCP-1 and stimulates invasion of monocytes into the vascular wall. In the present study, we observed that endotoxin-induced U-937 cell binding was blocked by scavengers of superoxide. Our observations therefore suggest that increased production of superoxide could, in part, mediate the proatherogenic effects of low-level endotoxin in human blood vessels.

TLR-4 has been proposed to mediate endotoxin signaling in humans. The TLRs are a family of evolutionarily conserved pattern-recognition receptors, first described in Drosophila, which function in innate immune recognition of pathogen-associated molecules, including endotoxin. TLR-4 is a transmembrane protein whose cytoplasmic tail exhibits homology with the IL-1 receptor. Cells from TLR-4–deficient mice, caused by a missense mutation in the TLR-4 coding sequence, failed to become activated in response to endotoxin. TLR-4 mRNA expression in human macrophages was upregulated by oxidized LDL, suggesting synergy between hyperlipidemia and endotoxia. Very recently, humans with the Asp299Gly TLR-4 polymorphism, which is associated with attenuated endotoxin responsiveness, were found to have a decreased risk of development of atherosclerosis. Thus, TLR-4 might be a key receptor in the development of inflammation and atherosclerosis. TLR-4 is known to be expressed in monocytes/macrophages and other types of immune cells and was detected in human microvascular and coronary artery endothelial cells and cardiac myocytes. TLR-4 has also been recently identified in macrophages infiltrating human atherosclerotic lesions obtained at autopsy. However, TLR-4 was not detected in resident vascular cells of the tissues examined in the latter study. In contrast, our intact HVs showed evidence of TLR-4 expression. Our findings therefore suggest that TLR-4 is constitutively expressed in HSV, which might explain the potent proinflammatory effects of endotoxin observed in this study. Considering that saphenous veins in vivo are typically free of atherosclerosis at the time of bypass surgery, the proinflammatory effect of low-level endotoxin is probably insufficient to induce atherosclerosis in the absence of other proatherosclerotic factors. Rather, endotoxin likely interacts with other factors, eg, hemodynamic factors and other humoral mediators, to induce atherosclerosis.

In summary, we have demonstrated that low levels of endotoxin induce proinflammatory activation of intact human blood vessels. Endotoxin-induced release of IL-8 and induction of monocyte binding are blocked by atorvastatin, suggesting a beneficial effect of statins unrelated to cholesterol lowering. Also, we have demonstrated the presence of the endotoxin receptor TLR-4 in HVs. These proinflammatory effects of very low levels of endotoxin on intact human blood vessels suggest a putative link between chronic or intermittent subclinical infection and increased risk of atherosclerosis. Moreover, our findings suggest that amelioration of chronic infections associated with low level endotoxia and/or therapy directed against the vascular effects of endotoxin might be a promising means of preventing atherosclerosis in humans.

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