Superoxide mediates endotoxin-induced platelet-endothelial cell adhesion in intestinal venules

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Submitted 8 April 2002; accepted in final form 3 October 2002

Cerwinka, Wolfgang H., Dianne Cooper, Christian F. Krieglstein, Chris R. Ross, Joe M. McCord, and D. Neil Granger. Superoxide mediates endotoxin-induced platelet-endothelial cell adhesion in intestinal venules. Am J Physiol Heart Circ Physiol 284: H535–H541, 2003. First published October 10, 2002; 10.1152/ajpheart.00311.2002.—Platelets have been implicated in the pathogenesis of different diseases of the vascular system, including atherosclerosis, sepsis, and ischemia-reperfusion injury; however, relatively little is known about the factors that regulate the interactions between circulating platelets and the vessel wall. The objective of this study was to define the contribution of superoxide to LPS-induced platelet-endothelial cell (P/E) adhesion in murine intestinal venules. The adhesion of rhodamine-6G-labeled murine platelets was monitored by intravital fluorescence microscopy. Four hours after LPS administration in control [wild-type (WT)] mice, an ~10-fold increase in P/E adhesion was detected. This response did not result from LPS-induced platelet activation. The LPS-induced P/E adhesion was greatly attenuated in NAD(P)H oxidase-deficient mice and in WT mice rendered neutropenic with anti-neutrophil serum, whereas the response was unchanged in WT mice receiving a CD18 blocking MAb or in CD18-deficient mice. A chimeric form of MnSOD that exhibits the binding properties of extracellular SOD also attenuated the LPS-induced response in WT mice. These findings indicate that neutrophil-derived superoxide plays a major role in the modulation of endotoxin-induced P/E adhesion.

endotoxemia; neutrophils; postcapillary venules

SEVERAL SYSTEMIC (e.g., atherosclerosis, septic or hemorrhagic shock) and regional (ischemia-reperfusion [I/R]) circulatory disorders are associated with a vascular system that assumes either a proinflammatory and/or a prothrombogenic phenotype. While the molecular and biochemical events that underlie these phenotypic changes remain poorly understood, there is now a large body of evidence that implicates an accelerated production of reactive oxygen species (ROS) as a key initiating event that leads to the enhanced recruitment of inflammatory cells in these pathological states. A direct linkage between ROS and leukocyte-endothelial cell (L/E) adhesion has been established in different experimental models of regional (14) and systemic (1) I/R, hypercholesterolemia (35), and sepsis (13). For example, mutant mice that either overexpress CuZnSOD (14) or are deficient in p47phox (a regulatory component of NAD(P)H oxidase) (35) exhibit a significant attenuation of L/E adhesion compared with their wild-type (WT) counterparts, suggesting that superoxide promotes leukocyte adhesion to vascular endothelium. On the basis of these observations and other studies, it has been proposed that superoxide (and/or secondarily derived ROS) either acts as a signaling molecule that initiates the expression and/or activation of endothelial cell adhesion molecules, or it inactivates nitric oxide (NO), a well-established endogenous inhibitor of L/E adhesion (11).

Although relatively little is known about the chemical mediators of platelet-endothelial cell (P/E) adhesion in vivo, there is a large body of data derived from in vitro models of P/E adhesion that support a potential role for superoxide. SOD has been shown to inhibit thrombin-induced P/E adhesion and platelet aggregation (32, 33). Similarly, the aggregation of human platelets that is induced by either anoxia/reoxygenation (25) or oxidized low-density lipoproteins (23) is profoundly reduced in the presence of SOD (17, 34). There is also evidence that implicates superoxide as a mediator of the heterotypic adhesion of platelets to circulating leukocytes after I/R (16).

Recently, the technique of intravital videomicroscopy has been employed in several laboratories to monitor and quantify the interactions of fluorescently labeled platelets with endothelial cells of postcapillary venules in inflamed tissue (8, 15, 20, 21). These studies have provided novel insights about the contribution of specific adhesion glycoproteins expressed on the surface of platelets and/or endothelial cells in mediating
the P/E adhesion induced in venules by bacterial endotoxin (LPS) (15) or I/R (20). In addition, the intravital microscopic studies have revealed a major role for NO (17) and cGMP (21) in modulating the P/E adhesion in intestinal venules induced by either LPS or I/R. This ability of NO to modulate P/E adhesion in vivo has been linked to cGMP-dependent signaling pathways (27); however, it is often speculated that a principal biological action of NO that leads to attenuated P/E adhesion is the scavenging of superoxide (3). Although in vitro studies support the contention that NO is a highly efficient scavenger of superoxide, the assumption that superoxide is a quantitatively significant modulator of P/E adhesion in vivo has not been directly or systematically addressed. Hence, the overall objectives of this study were 1) to evaluate the contribution of superoxide to the modulation of P/E adhesion in LPS-stimulated intestinal venules, and 2) to assess the potential role of neutrophils as a source of the superoxide that mediates LPS-induced P/E adhesion. The findings of this study provide strong support for neutrophil-derived superoxide as a mediator of endotoxin-induced P/E adhesion and indicate that activation of endothelial cells, but not platelets, is responsible for the proadhesive action of LPS.

MATERIALS AND METHODS

Animals. WT mice (C57BL/6), CD18-deficient hypomorphic mutants (C57BL/6-Itgb2<tm1bay>), and breeder stocks for SOD transgenic (TG) (C57BL/6-TgN(SOD1) 10Cje:SOD) mice were obtained from Jackson Laboratory (Bar Harbor, ME). The SOD TG mice were identified by Tg-specific genomic Southern blot with an excitation filter block with an excitation filter for 510–560 nm, a dichroic mirror for 580 nm, and a barrier filter for 590 nm (G-2A, Nikon). The images were then recorded on a video recorder (BR-S801MU, JVC) for off-line evaluation. The intestinal loop was scanned for three to five venules (mean = 4.6), and each was recorded for 1 min.

Video analysis. Venular diameter (mean = 35 μm) was measured, and venular length set at 200 μm. Platelets were classified according to their interaction with the venular wall as either free flowing, rolling, or adherent (4). Firmly adherent platelets were classified according to the duration of their immobility on the venular wall, i.e., >2 s, >2 s <30 s, and >30 s. Platelet adherence was expressed as the number of cells per square millimeter of venular surface, calculated from diameter and length, assuming cylindrical vessel shape (20). In some experiments, estimates of pseudoshear rate in venules were obtained using measurements of venular diameter (Dv) and the maximal velocity of flowing platelets (Vpmax) according to the following formulation: pseudoshear rate = (Vpmax/1.6)/Dv × 8 (39).

In an additional group of experiments, leukocyte and platelet interactions with the venular wall were examined simultaneously. For these experiments, platelets were isolated as described above with the following modifications. Washed platelets were incubated with the fluorescent dye carboxyfluorescein diacetate succinimidyld ester (90 μM, Molecular Probes; Eugene, OR) for 10 min at room temperature. The platelets were then centrifuged for 10 min at 550 g, resuspended in 500 μl PBS, and protected from light until use. Rhodamine-6G (0.02%) was administered via the jugular vein for visualization of leukocytes. Platelet and leukocyte adhesion was quantified simultaneously within venules using a Nikon filter block (B) with an excitation filter for 470–490 nm, a dichroic mirror for 510 nm, and a barrier filter of 520 nm. Platelet adhesion was quantified as described
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Adherent leukocytes were classified as those remaining stationary on the venular wall for >30 s. This protocol was used in a group of LPS-treated CD18-deficient mice and in WT mice receiving LPS as well as a blocking MAb against CD18 (see Experimental protocols).

Experimental protocols. All animals except platelet donors were fasted for 24 h. An LPS dose of 0.5 mg/kg ip and an incubation period of 4 h was employed in all experimental groups. This dose of LPS has been previously shown to activate murine intestinal endothelial cells, as manifested by a 15-fold increase in P-selectin expression and a >20-fold increase in E-selectin expression (6). Sham-treated animals received 0.5 ml ip saline instead of LPS. To determine the role of platelets and endothelial cells in LPS-induced P/E interactions, either platelet donors, platelet recipients, or both were exposed to LPS. Some LPS-treated mice received a chimeric form of SOD, a fusion protein consisting of the mature human MnSOD sequence, followed by the 26 COOH-terminal residues of human extracellular SOD (ecSOD) (10, 24). This chimeric SOD behaves much like ecSOD in that it avidly binds to the surface of vascular endothelial cells. Native MnSOD (E. coli, Sigma) was used as a control. Either chimeric SOD or MnSOD was administered via the left jugular (South Natick, MA) infusion pump, yielding a 15-fold increase in P/E adhesion (Fig. 1). Significant for both the chimeric SOD and MnSOD groups using Scheffe’s (post hoc) test, these differences became significant for either >2 s (Fig. 1A) or >30 s (Fig. 1B) were significantly increased at 4 h after LPS treatment. Sham-treated animals exhibited a relatively small number of temporarily adherent platelets (>2 s); however, no permanently adherent platelets (>30 s) could be detected. For all subsequent experiments, only the data for P/E adhesion >2 s and >30 s/mm² are presented.

Platelet donor and platelet recipient. To determine the contribution of platelets versus endothelial cells in LPS-induced P/E interactions, either platelet donors, platelet recipients, or both were exposed to LPS. When the mice providing the platelets (donors) were not exposed to LPS, but the recipient mice did receive LPS (4 h previously), a significant increase in P/E adhesion was noted compared with sham animals (neither donor nor recipients received LPS; Fig. 2). However, when the mice serving as platelet donors were treated with LPS, but the recipient (receiving the LPS-treated platelets) mice were not, the P/E adhesion response was similar to sham-treated animals, i.e., minimal P/E adhesion was observed. Treatment of both platelet donor and recipient mice with LPS produced a response similar to that observed when the recipients alone were treated with LPS. These experiments revealed that LPS-induced P/E adhesion is not dependent on direct activation of platelets by LPS.

Superoxide dismutase. To assess the role of superoxide in LPS-induced P/E adhesion, we employed SOD TG mice overexpressing CuZnSOD, an isoform of the enzyme that is distributed in the cytoplasm. When SOD TG mice were challenged with LPS, the response did not differ from WT mice that were treated with LPS (Fig. 3). Similarly, intravenous administration of native MnSOD did not attenuate LPS-induced P/E adhesion. However, when ecSOD-like chimeric SOD was administered intravenously, the more prolonged (>30 s) adhesion of platelets induced by LPS was abrogated. Whereas a difference was not detected between the chimeric SOD and MnSOD groups using Scheffe’s post hoc test, these differences became significant for both the >30 s (P = 0.041) and >2 s (P = 0.33).

Results

P/E interactions. LPS treatment of WT mice did not significantly alter the flux of rolling platelets in intestinal venules (data not shown), nor was there a difference in pseudoshear rates between venules of sham (473 ± 53 s⁻¹) and LPS-treated (441 ± 72 s⁻¹) WT mice (Fig. 1). However, the number of platelets that were firmly adherent for either >2 s (Fig. 1A) or >30 s (Fig. 1B) were significantly increased at 4 h after LPS treatment. Sham-treated animals exhibited a relatively small number of temporarily adherent platelets (>2 s); however, no permanently adherent platelets (>30 s) could be detected. For all subsequent experiments, only the data for P/E adhesion >2 s and >30 s/mm² are presented.

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platelet adhesion data when a Fisher's post hoc test was applied. These findings suggest that the superoxide that mediates LPS-induced P/E adhesion is generated at or near the surface of cells.

**Neutrophils.** To determine whether neutrophils are a major source of the superoxide that mediates LPS-induced P/E adhesion, mice were rendered neutropenic using the anti-neutrophil MAb RB68C5 (Fig. 4). This mAb yielded a 90% reduction in blood neutrophils without affecting the circulating counts of platelets, lymphocytes, and monocytes. These neutropenic mice exhibited a significantly blunted P/E adherence (but not rolling) response after challenge with LPS. To assess the importance of leukocyte adhesion to this neutrophil-dependent, LPS-induced P/E adhesion response, experiments were performed in mice genetically deficient in the leukocyte adhesion glycoprotein CD11/CD18, which has been previously implicated as a mediator of LPS-induced adhesion in the cat mesentery (13). These LPS-challenged CD18-deficient mice exhibited P/E adhesion responses to LPS that were comparable with those noted in WT mice (Fig. 4). In view of this response of CD18-deficient mice to LPS, additional experiments were performed to determine whether LPS-induced leukocyte adhesion in the mouse intestine is CD18 dependent. These experiments revealed no significant difference in leukocyte adhesion between LPS-challenged CD18-deficient mice (566.4 ± 59.2 leukocytes/mm²) and LPS-treated WT mice (741.8 ± 53.7 leukocytes/mm², respectively) nor was it significantly reduced in LPS-challenged WT mice that received a CD18 blocking MAb (534.9 ± 64.4 leukocytes/mm²). The magnitude of platelet adhesion elicited by LPS also did not differ between these experimental groups.

**NADPH oxidase deficiency.** To further assess the role of leukocytes as a potential source of superoxide that mediates LPS-induced P/E adhesion, experiments were performed in mice genetically deficient in p47phox, a regulatory subunit of neutrophilic NAD(P)H oxidase (Fig. 5). Sham-treated p47phox-deficient mice exhibited an insignificant level of P/E adhesion. Although the LPS-induced P/E adhesion elicited in p47phox heterozygous mice was no different from that observed in WT mice, a significantly attenuated P/E adhesion response to LPS was noted in p47phox homozygous mice. Similarly, the number of adherent platelets was significantly different between LPS-treated p47phox heterozygous and LPS-treated homozygous mice. The attenuation of P/E adhesion observed in p47phox homozygous mice was comparable with that observed in WT mice rendered neutropenic (Fig. 4).

**DISCUSSION**

It is now well recognized that platelets can adhere to microvascular endothelial cells when tissues are ex-
posed to stimuli such as I/R (20), cytokines (9), calcium ionophores (8), and bacterial endotoxins (15). These adhesive interactions between platelets and endothelial cells have been implicated in the vascular responses to tissue injury, including the recruitment and activation of leukocytes and thrombogenesis (7, 31). The recognition that P/E adhesion may contribute to the initiation and/or progression of tissue injury has fueled an interest in defining the adhesion molecules that mediate these cell-cell interactions as well as the chemical factors that enable tissues to elicit P/E adhesion. Both NO and superoxide are considered to be potentially important modulators of homotypic platelet aggregation as well as the adhesion of platelets to endothelial cells, with superoxide promoting and NO inhibiting the platelet adhesion responses (12, 19, 26, 30, 32). Recent studies on the intestinal and mesenteric microcirculation have implicated specific adhesion glycoproteins (glycoprotein Ib-α) as well as endothelial cell-derived NO as participants in the P/E adhesion response elicited by LPS (4, 15, 20). While the inhibitory action of endogenous and exogenous NO on LPS-induced P/E adhesion can be partly attributed to activation of cGMP-dependent signaling pathways (21), it has been proposed that NO may also exert this beneficial effect by scavenging superoxide. The role of superoxide in modulating LPS-induced P/E adhesion was addressed in the present study.

An issue that remained unresolved from previous studies of LPS-induced P/E adhesion is whether direct platelet activation by LPS is required for the adhesion response. This issue was addressed in the present study by comparing the P/E adhesion responses when either platelet donors, platelet recipients, or both were exposed to LPS. Our findings indicate that direct activation of platelets by LPS is not a requirement for the observed P/E adhesion response. Furthermore, these experiments indicate that endothelial cells and/or other cell populations play a more important role in eliciting P/E adhesion in LPS-treated mice. Our previous work indicates that the dose of LPS employed in these experiments result in significant endothelial cell activation as evidenced by a 15-fold increase in P-selectin expression and a >20-fold increase in E-selectin expression (6). This does not exclude a role for other cell types (e.g., leukocytes) that are also known to be activated by LPS (28). Indeed, our results strongly implicate neutrophils in the modulation of P/E adhesion induced by LPS.

Many compounds released by activated neutrophils may act as platelet agonists (e.g., superoxide, hydrogen peroxide) or antagonists (ADPases); conversely, platelets can release factors that may either inhibit (soluble P-selectin, NO) or activate (oxygen radicals, leukotrienes, thromboxane A2) neutrophils (39). Because LPS is known to directly activate neutrophils by both tran-

Fig. 4. Neutrophils contribute to endotoxin-induced P/E adhesion. The effects of LPS (0.5 mg/kg in 0.5 ml saline ip, 4-h incubation) on P/E adhesion in intestinal venules of WT mice rendered neutropenic with anti-neutrophil serum and in CD18-deficient (CD18−/−) mice are shown. A: mean responses of platelets adherent for >2 s; B: mean responses of platelets adherent for >30 s. Brackets indicate significant differences, P < 0.05 (ANOVA and Scheffe’s test).

Fig. 5. NAD(P)H oxidase-derived superoxide mediates endotoxin-induced P/E adhesion. The effects of LPS (0.5 mg/kg ip, 4-h incubation) on P/E adhesion in intestinal venules of WT mice, in mice deficient in p47phox (p47phox−/−), and in their heterozygous (p47phox+/−) littermates are shown. A: mean responses of platelets adherent for >2 s; B: mean responses of platelets adherent for >30 s. Brackets indicate significant differences, P < 0.05 (ANOVA and Scheffe’s test).
scription-dependent and -independent (e.g., complement activation) mechanisms, it is possible that neutrophils contribute to LPS-induced P/E adhesion by producing platelet agonists. This possibility was assessed by rendering animals neutropenic using a mouse neutrophil-specific mAb. The results from these experiments strongly implicate neutrophils as modulators of LPS-induced P/E adhesion. The potential role of leukocyte (including neutrophil) adhesion in the neutrophil-mediated, LPS-induced P/E adhesion was addressed using two strategies: 1) mice genetically deficient in the leukocyte adhesion glycoprotein CD11/CD18, and 2) WT mice receiving a blocking mAb directed against CD18. Although neither genetic deletion or immunoneutralization of CD18 resulted in a significant reduction in LPS-induced platelet adhesion, the adhesion of leukocytes induced by LPS also was not reduced in either instance. The latter observation suggests that unlike in the cat mesentery, where a CD18 mAb significantly blunts LPS-induced leukocyte adhesion (13), other adhesion molecules mediate this response in mouse intestinal venules. Because our experiments did not resolve the importance of L/E adhesion to the LPS-induced platelet adhesion response, the possibility remains that platelets bind within venules after LPS stimulation as part of a leukocyte-platelet aggregate. Clear identification of the molecular determinants of LPS-induced leukocyte adhesion is needed to resolve this issue.

The possibility that superoxide may mediate the neutrophil-dependent, LPS-induced P/E adhesion response was addressed using different strategies to elevate SOD activity in different compartments of LPS-treated tissues. SOD TG mice were used to assess the influence of an elevated cytosolic SOD activity, whereas intravenous native MnSOD and a chimeric SOD with ecSOD-like binding properties (24) were used to define the influence of elevated plasma and cell surface SOD activities, respectively. We found that increasing SOD activity in the cytosol (SOD TG mice) or plasma (mice receiving native MnSOD intravenously) did not afford protection. These same interventions have been shown to significantly attenuate L/E adhesion in different models of acute inflammation, including LPS treatment (14, 36, 37). However, we did observe a significant reduction in LPS-induced P/E adhesion in WT mice receiving a “designer” chimeric SOD genetically engineered to yield a human MnSOD molecule fused to a positively charged COOH-terminal “tail” consisting of a sequence of 26 amino acids that comprise the heparin-binding domain of human ecSOD (24). This “tail” enables the chimeric SOD to bind to heparin-like proteoglycans on vascular cell surfaces. Recent studies have shown that this chimeric SOD protects the myocardium against warm or cold ischemia at doses nearly two orders of magnitude lower than those required using a native human MnSOD (24). The ability of chimeric SOD to blunt LPS-induced P/E adhesion suggests that the superoxide that mediates LPS-induced P/E adhesion is generated at or near the surface of cells such as neutrophils, platelets, and/or endothelial cells. Our data do not allow us to define which cell population(s), i.e., platelets, endothelial cells, or both, were affected by the chimeric SOD to yield a reduction in P/E adhesion.

NADPH oxidase is a cell membrane-associated enzyme that enables neutrophils and other cell populations (including platelets and endothelial cells) to produce superoxide (2, 18, 29). Because our study implicated both neutrophils and superoxide in the P/E adhesion induced by LPS, we addressed the possibility that neutrophils are the source of superoxide in this experimental model of inflammation. With the use of mice genetically deficient in p47^phox, a key regulatory subunit of NADPH oxidase, we demonstrated a significantly blunted LPS-induced P/E adhesion response compared with both WT mice and their heterozygous (superoxide producing) littermates. Our observation that the magnitude of the inhibitory effect on P/E adhesion seen in the LPS-treated NADPH oxidase-deficient mice was comparable with that noted in LPS-treated neutropenic animals strongly suggests that neutrophils are the major source of the NADPH oxidase that contributes to the superoxide-mediated response. Nonetheless, we cannot exclude the possibility that either platelet-associated NADPH oxidase, endothelial cell-associated NADPH oxidase, or both also contribute to LPS-induced P/E adhesion.

In conclusion, the results of this study implicate superoxide as an important mediator of the P/E adhesion that is elicited in intestinal venules by E. coli endotoxin. It is likely that superoxide represents one of several factors that contribute to the prothrombogenic state that is associated with endotoxemia. When considered in conjunction with our recent report (4) that describes a protective role for endogenous and exogenous NO in LPS-induced P/E adhesion, it appears that an imbalance between superoxide and NO may be a critical initiating event that promotes P/E adhesion after LPS challenge. The findings of this study also suggest that the superoxide that mediates LPS-induced P/E adhesion is generated by the neutrophil membrane-associated enzyme NAD(P)H oxidase. Direct activation of platelets by LPS is not required for this superoxide-mediated, neutrophil-dependent response. Whether or not neutrophil adhesion to venular endothelium is required for this response remains unclear. Additional work is needed to define more precisely the molecular and cellular basis for the P/E adhesion that is mediated by superoxide.

The authors are grateful to Janice Russell for assistance with the experiment procedures and preparation of illustrations.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-26441.

REFERENCES


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