Role of superoxide in hemorrhagic shock-induced P-selectin expression

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Akgür, Feza M., Mark F. Brown, Gazi B. Zibari, John C. McDonald, Charles J. Epstein, Christopher R. Ross, and D. Neil Granger. Role of superoxide in hemorrhagic shock-induced P-selectin expression. Am J Physiol Heart Circ Physiol 279: H791–H797, 2000.—Superoxide has been implicated in the regulation of endothelial cell adhesion molecule expression and the subsequent initiation of leukocyte-endothelial cell adhesion in different experimental models of inflammation. The objective of this study was to assess the contribution of oxygen radicals to P-selectin expression in a murine model of whole body ischemia-reperfusion, i.e., hemorrhage-resuscitation (H/R), with the use of different strategies that interfere with either the production (allopurinol, CD11/CD18-deficient or p47phox−/− mice) or accumulation (intravenous superoxide dismutase (SOD), mutant mice that overexpress SOD) of oxygen radicals. P-selectin expression was quantified in different regional vascular beds by use of the dual-radiolabeled monoclonal antibody technique. H/R elicited a significant increase in P-selectin expression in all vascular beds. This response was blunted in SOD transgenic mice and in wild-type mice receiving either intravenous SOD or the xanthine oxidase inhibitor allopurinol. Mice genetically deficient in either a subunit of NADPH oxidase or the leukocyte adhesion molecule CD11/CD18 also exhibited a reduced P-selectin expression. These results implicate superoxide, derived from both xanthine oxidase and NADPH oxidase, as mediators of the increased P-selectin expression observed in different regional vascular beds exposed to hemorrhage and retransfusion.

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Both oxygen radicals and leukocytes have been implicated in the pathogenesis of ischemia-reperfusion (I/R) injury (10, 11, 31). Evidence supporting a role for oxygen radicals is provided by studies demonstrating diminished I/R injury in animals treated with agents that either blunt the production of or scavenge oxygen radicals or in mutant mice that overexpress an oxygen radical-scavenging enzyme, such as superoxide dismutase (SOD) (8, 10, 11, 14, 31). Multiple experimental strategies have also been used to invoke a role for leukocytes in I/R injury. Animals rendered neutropenic or that receive monoclonal antibodies that prevent leukocyte-endothelial cell adhesion (LECA) (5, 10, 12, 23) as well as mice that are genetically deficient in adhesion molecules that mediate LECA (5, 12, 23) all exhibit a diminished injury response to I/R. Although the ability of both oxygen radical- and leukocyte-directed interventions to blunt I/R injury suggests that at least two distinct mechanisms are involved in the injury process, there is evidence supporting a link between the two mediators (oxygen radicals and leukocytes) of I/R injury (10, 11, 31). Published reports describing the attenuation of I/R-induced LECA in animals treated with radical-scavenging enzymes (14, 19, 29) or peptide inhibitors of neutrophilic NADPH oxidase (17) or in mutant mice that overexpress SOD (6, 14) suggest that oxygen radicals contribute to reperfusion injury by initiating the recruitment and activation of adherent leukocytes.

LECA is regulated by a number of factors, including cell adhesion molecules (CAMs) expressed on the surfaces of leukocytes and endothelial cells (5, 12, 23). Although it has been shown that both leukocyte and endothelial CAMs are upregulated in tissues exposed to I/R, the mechanisms that contribute to this increased expression of adhesion glycoproteins remain poorly defined. P-selectin, which is the first endothelial CAM that is upregulated after I/R (5, 12, 23), appears to be under the control of oxygen radicals. Both in vitro (25) and in vivo (4, 9) experiments have revealed that superoxide and hydrogen peroxide are potent stimuli for the rapid upregulation of P-selectin on vascular endothelial cells. However, the relevance of these observations to the condition of I/R, wherein oxygen radical production is linked to LECA, remains uncertain. It is conceivable that I/R-induced oxygen radical production, resulting from either the activation of endo-
thelial xanthine oxidase or neutrophilic NADPH oxidase, elicits the expression of P-selectin. The objective of this study was to test this possibility in a murine model of whole body IR, i.e., hemorrhage-resuscitation (H/R), with the use of both oxygen radical- and leukocyte-directed strategies to interfere with either the production or accumulation of oxygen radicals.

**MATERIALS AND METHODS**

**Monoclonal antibodies.** The monoclonal antibodies (MAbs) used for the in vivo assessment of P-selectin were RB40.34, a rat immunoglobulin G1 (IgG1) against mouse P-selectin (Pharmigen, San Diego, CA), and P-23, a nonbinding murine IgG1 directed against human P-selectin.

**Radioiodination of monoclonal antibodies.** RB40.34 and P-23 were labeled with 125I and 131I (NEN Life Sciences Products, Boston, MA), respectively, with the use of the iodogen method (6). In brief, 250 μg of protein were incubated with 250 μCi of sodium iodine-125 (or sodium iodine-131) and 125 μg of sodium iodide at 4°C for 5 min. Phosphate-buffered saline (PBS) (pH 7.4) was added to bring the volume to 2.5 ml. Thereafter, the radiolabeled MAb was separated from free 125I or 131I by gel filtration on a Sephadex PD-10 column (Pharmacia & Upjohn, Uppsala, Sweden). The column was equilibrated and then eluted with PBS containing 1% bovine serum albumin. Two fractions of 2.5 ml were collected, the second of which contained the radiolabeled MAb. Absence of free 125I or 131I was ensured by extensive dialysis of the protein-containing fraction. Radiolabeled MAbs were stored at 4°C until use.

**Animal procedures.** All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Medical Center.

**Calculation of P-selectin expression.** The 125I (binding MAb) and 131I (nonbinding MAb) activities in different organs and a 50-μl plasma sample were counted in a 14800 Wizard 3 gamma-counter (Wallac, Turku, Finland), with automatic correction for background activity and spillover. A 4-μl aliquot of the preinjection mixture of labeled MAbs was assayed to determine total injected activity of each labeled MAb. The tube used to mix the MAbs and the infusion syringe were likewise counted, and their activities were subtracted from the total levels. P-selectin expression was determined by subtracting the accumulated activity of the nonbinding MAb from that of the binding MAb activity and was expressed as nanograms of MAb per gram of tissue.

**Experimental protocols.** Mice were anesthetized with intraperitoneal ketamine (150 mg/kg) and xylazine (7.5 mg/kg). The left femoral artery was catheterized through an inguinal incision by use of a PE-50 catheter with a PE-10 tip. The catheter was connected to a transducer attached to a pressure monitor (BP-1; World Precision Instruments, Saratoga, FL) for blood pressure measurements. The catheter and transducer were primed with normal saline containing 10 U/ml heparin. The maximum amount of heparin introduced by the end of the experiments with infusion of shed blood to the animal was 3 U (0.1–0.15 U/g). Blood withdrawal and arterial pressure measurements were performed through the same arterial line. The mice were bled by withdrawing blood into the 1-ml syringe over a 5-min period until a predetermined mean arterial pressure (MAP) of 30 mmHg was reached. MAP was maintained at 30 mmHg for 45 min by the withdrawal or reinfusion of shed blood. Body temperature was monitored through a rectal thermistor and was maintained at 37°C with the aid of a heating pad. At the end of the 45-min hemorrhagic shock period, animals were resuscitated by infusion of lactated Ringer solution with a volume corresponding to 50% of the shed blood volume. Thereafter, shed blood was infused. Resuscitation with lactated Ringer solution plus shed blood was performed over a 10-min period. In wild-type (control) mice, the bleed-out volume (in ml/100 g body wt) was 2.62 ± 0.12. The mean values for bleed-out volume in all experimental groups did not differ significantly from the control group. The initial (prehemorrhage) arterial blood pressure in wild-type mice was 76.3 ± 4.2 mmHg, and the postresuscitation (5 min) blood pressure was 77.5 ± 2.1 mmHg. These values were not significantly different in the other experimental groups, except in the p47phox−/− mice, which had initial and postresuscitation blood pressures of 97.8 ± 4.8 and 104.4 ± 4.9 mmHg, respectively.

Constitutive levels of P-selectin expression were determined in lung, heart, liver, kidney, mesentery, stomach, small bowel, and colon of C57BL/6 (wild-type) mice (n = 11). P-selectin expression was also determined in the same organs at 5 h after resuscitation of hemorrhagic shock in 1) C57BL/6 mice (n = 12), 2) SOD-Tg mice (n = 5), 3) SOD-NonTg mice (n = 3), 4) C57BL/6 mice treated with intravenous SOD (n = 5), 5) C57BL/6 mice receiving allopurinol (n = 5), 6) CD11/CD18-deficient mice (n = 5), and 7) NADPH oxidase-deficient (p47phox−/−) mice (n = 8). SOD (Sigma, St. Louis, MO) was infused during resuscitation with the lactated Ringer solution (8,000 U/kg) (14). Allopurinol (Sigma) was administered orally (50 mg/kg) for 2 days before surgery. An additional dose of allopurinol (50 mg/kg) was administered 1 h before hemorrhagic shock (19).
Fig. 1. Effects of hemorrhage-resuscitation (H/R) on P-selectin expression in untreated and superoxide dismutase (SOD; iv)-treated wild-type mice as well as in SOD-overexpressing mutants (SOD-Tg) or their normal littermates (SOD-nonTg) in lung, heart, liver, and kidney (A) and mesentery, stomach, small bowel, and colon (B). *P < 0.01 compared with constitutive levels and †P < 0.001 relative to H/R alone; no. of mice (n) = 5 in each group. MAb, monoclonal antibody.
Fig. 2. Effects of H/R on P-selectin expression in untreated and allopurinol-treated wild-type mice as well as in mutant mice genetically deficient in CD11/CD18 (CD11/CD18 def). A: summary of results obtained for lung, heart, liver, and kidney. B: findings for mesentery, stomach, small bowel, and colon. *P < 0.01 compared with constitutive levels and †P < 0.01 compared with H/R alone; n = 5 in each group.
RESULTS

Figure 1, A and B, summarizes the changes in P-selectin expression induced by H/R in different organs of untreated and SOD-treated (iv) wild-type (WT) mice as well as in SOD-overexpressing mutants (SOD-Tg) or their normal littermates (SOD-nonTg). In all organs studied, H/R elicited a profound increase in P-selectin expression. Treatment of WT mice with intravenous SOD significantly attenuated the H/R-induced P-selectin expression. A similar attenuation of P-selectin expression after H/R was noted in SOD-Tg but not in SOD-nonTg mice. These findings indicate that either administration of exogenous SOD to WT mice or genetic overexpression of SOD results in a significant attenuation of H/R-induced P-selectin expression.

Figure 2, A and B, illustrates the H/R-induced changes in P-selectin expression observed in different vascular beds of untreated and allopurinol-treated WT mice and in mice that are genetically deficient in the leukocyte adhesion molecule CD11/CD18 (CD18 deficient). The xanthine oxidase inhibitor allopurinol significantly attenuated the H/R-induced expression of P-selectin. Mutant mice that are genetically deficient in CD11/CD18 also exhibited a profound attenuation of the H/R-induced P-selectin response. The attenuation of P-selectin expression was significantly greater for CD18-deficient mice than for allopurinol-treated mice. These findings indicate that both xanthine oxidase and activated neutrophils may contribute to the upregulation of P-selectin that is elicited by H/R.

Figure 3 illustrates the H/R-induced changes in P-selectin expression observed in the intestinal vascular lumens of the small intestine. The H/R-induced increment in P-selectin expression was significantly lower in the intestines of p47phox−/− mice compared with their WT counterparts. These experiments indicate that NADPH oxidase contributes to ~35% of the P-selectin expression elicited by H/R in the small intestine. Although some other tissues (e.g., mesentery and colon) also exhibited a significantly lower expression of P-selectin in p47phox−/− mice, most tissues studied did not.

DISCUSSION

There is a large body of evidence that implicates activated neutrophils in the pathogenesis of H/R injury (1–3, 13, 21). Compelling evidence for the contribution of neutrophils in this disease process is provided by studies showing attenuated injury in animals that are treated with CAM MAbs (20, 26, 30). These findings suggest that an increased expression of adhesion molecules on leukocytes and/or endothelial cells is a critical component of the pathological process. Although several studies have demonstrated upregulation of soluble CAMs in humans with H/R (27, 28), it remains uncertain which endothelial CAM is expressed (and by how much) after H/R and what mechanism(s) accounts for this response. In the present study, we demonstrate that P-selectin, an endothelial receptor for rolling leukocytes, is expressed more intensely in different vascular beds after exposure to H/R. Furthermore, evidence is provided that implicates superoxide as an important mediator of the H/R-induced P-selectin expression.

The magnitude of the increased P-selectin expression induced by H/R varied between vascular beds, with the kidneys and liver exhibiting the smallest and largest relative changes, respectively (1.5× and 200× control, respectively). Most vascular beds exhibited three- to fivefold increases in P-selectin expression over constitutive (basal) levels. P-selectin expression in the intestinal vascular bed increased approximately fivefold after H/R. This response is comparable with the approximately fourfold increase observed in murine small intestine exposed to 20 min of local ischemia (complete occlusion of the superior mesenteric artery) and 5 h of reperfusion (6). However, the P-selectin responses of all vascular beds to H/R are significantly lower than the 15- to 75-fold increases in P-selectin expression measured in murine tissues after high-dose endotoxin treatment (7). Thus it appears that although the P-selectin upregulation elicited by H/R is substantial, it represents only a fraction of the maximal capacity of endothelial cells to express this adhesion molecule.

Superoxide has been implicated in the regulation of endothelial CAM expression and the subsequent initiation of LECA (12). Exogenously generated superoxide has been shown to promote P-selectin expression on monolayers of cultured endothelial cells (25) and to elicit P-selectin-dependent rolling in intact postcapillary venules (4, 9). Furthermore, it has been shown that intravascular administration of either CuZn-SOD or Mn-SOD significantly attenuates the LECA that is elicited in venules by I/R (15, 19, 29) or exogenous platelet-activating factor (PAF) (18, 32), both of which

Statistics. Data among groups were compared with the use of an analysis of variance. The Tukey’s test was used for multiple comparisons. Significance was set at P < 0.01.
cause an oxidant stress in venules (7, 11). Similarly, it has been shown that the LECA induced by I/R in the hepatic microvasculature is significantly lower in mutant mice that overexpress CuZn-SOD compared with their WT counterparts (14). The results of the present study provide the first direct support for a role of superoxide in the induction of endothelial CAM expression after H/R. Using two different experimental strategies, i.e., transgenic mice that overexpress CuZn-SOD and WT mice receiving intravenous CuZn-SOD, we demonstrated that scavenging the superoxide anions generated in response to H/R significantly attenuates the accompanying enhancement of P-selectin expression. In the tissues studied, the inhibition of H/R-induced P-selectin expression was similar for CuZn-SOD transgenics and WT mice receiving exogenous SOD. This observation suggests that increased extracellular SOD is as effective as increased intracellular SOD in preventing the P-selectin upregulation, possibly reflecting a source of superoxide that is at, or near, the endothelial cell surface.

Studies on endothelial cell monolayers exposed to anoxia-reoxygenation (A/R) and postcapillary venules subjected to I/R have revealed a role for xanthine oxidase in producing the superoxide detected in postischemic (hypoxic) endothelial cells (15). The possibility that xanthine oxidase-derived superoxide mediates A/R- or I/R-induced LECA is supported by studies demonstrating an inhibitory effect of allopurinol on LECA that is comparable with that observed with SOD treatment (15). Our finding that allopurinol reduced H/R-induced P-selectin expression supports the possibility that xanthine oxidase contributes to the superoxide-mediated response. Xanthine oxidase could contribute to this response in two ways: 1) by directly generating the superoxide that elicits P-selectin biosynthesis and cell surface mobilization in postischemic endothelium and/or 2) by eliciting superoxide-mediated recruitment of leukocytes, which, in turn, generate superoxide fluxes (from NADPH oxidase) that result in the increased expression of P-selectin.

A role for leukocytes in mediating H/R-induced P-selectin expression is supported by our observation that mice that are genetically deficient in CD11/CD18 also exhibit an attenuated P-selectin response to H/R. The leukocyte adhesion molecule CD11/CD18 mediates the firm adhesion of neutrophils to postischemic vascular endothelium and is required for neutrophils to generate maximal levels of superoxide (15). Our findings in CD11/CD18-deficient mice suggest that adherent leukocytes may be the major source of superoxide that elicits the increased P-selectin expression after H/R. However, this possibility is not supported by experiments performed in NAPDH oxidase-deficient (p47phox−/−) mice (16), which exhibited a smaller decrement in H/R-induced P-selectin expression than either allopurinol-treated WT mice or CD11/CD18-deficient mice. Moreover, the attenuated P-selectin responses observed in p47phox−/− mice were limited to only a few vascular beds in the splanchnic circulation.

Our observation that both xanthine oxidase inhibition and prevention of LECA are effective in blunting the H/R-induced P-selectin response suggests that these two sources contribute to the response via a common pathway. It is possible, therefore, that the two sources are linked by a mechanism wherein superoxide generated from xanthine oxidase (and other sources) promotes the formation of mediators (e.g., PAF) that upregulate CD11/CD18 on leukocytes. The adherent leukocytes then generate substances (e.g., proteases) onto the surface of endothelial cells, which respond by synthesizing and expressing P-selectin. The novel data derived from the p47phox−/− mice indicate that adherent leukocytes are not a major source of the superoxide that modulates P-selectin after H/R. These findings suggest that therapeutic interventions directed toward prevention of the systemic inflammatory response to H/R should focus on scavenging superoxide or preventing LECA rather than on inhibiting the production of oxygen radicals.

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