Nitric oxide modulates gut ischemia-reperfusion-induced P-selectin expression in murine liver

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Nitric oxide modulates gut ischemia-reperfusion-induced P-selectin expression in murine liver. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H520–H526, 1998.—Reperfusion of ischemic intestine is associated with P-selectin-dependent adhesion of leukocytes in the liver microcirculation. The objective of this study was to define the contribution of nitric oxide (NO) to the enhanced P-selectin expression and increased leukocyte rolling elicited by gut ischemia-reperfusion (I/R). Leukocyte rolling (monitored by intravital microscopy) and P-selectin expression (measured using the dual-radio-labeled monoclonal antibody technique) were determined in mice after 15 min of superior mesenteric artery occlusion and 30 min of reperfusion. After 30 min of reperfusion, P-selectin expression was significantly increased in the liver, intestine, and lung, with a corresponding increase in the number of rolling leukocytes (in THV). The NO synthase inhibitor G-monomethyl-L-arginine exaggerated the gut I/R-induced increases in both rolling leukocytes and P-selectin expression (in liver and intestine), responses that were not detected with coadministration of L-arginine or in P-selectin-deficient mice. The NO donor diethylenetriamine/NO and L-arginine were both effective in attenuating the gut I/R-induced increases in rolling leukocytes (in THV) and P-selectin expression (in liver, intestine, and lung). These findings indicate that NO modulates gut I/R-induced recruitment of rolling leukocytes in THV via an action on P-selectin expression.

leukocyte-endothelial cell adhesion; nitric oxide synthase; lung; L-arginine.

Reperfusion of the ischemic liver and/or intestine is often associated with injury to the liver and lungs. A rate-limiting step in the pathogenesis of ischemia-reperfusion (I/R) injury in the liver and distant organs (such as the lung) is the adhesion of leukocytes to vascular endothelial cells. This contention is supported by studies demonstrating 1) an accumulation of leukocytes and tissue injury after I/R (8) and 2) an attenuated I/R-induced leukocyte recruitment and tissue injury in adhesion molecule-deficient mice or in animals receiving adhesion molecule-specific monoclonal antibodies (MAb) (1, 8, 12, 14). The results of the latter studies also suggest that the protective action of leukocyte adhesion-directed interventions can be demonstrated at different points in the leukocyte recruitment cascade, i.e., leukocyte rolling, adherence, and transendothelial migration. P-selectin-specific interventions, for example, have been shown to attenuate the leukocyte recruitment, tissue hypoxia, and hepatocellular necrosis in the liver (12) as well as the increased pulmonary vascular permeability (1) observed after intestinal I/R. On the basis of these observations it has been assumed that P-selectin expression is increased in the hepatic and pulmonary circulations after gut I/R and that this adhesion molecule mediates leukocyte rolling, which is a prerequisite for firm adhesion and transendothelial migration of leukocytes. Whether P-selectin expression increases in distant organs after gut I/R is unclear, and the factors that might contribute to such an increased expression of P-selectin remain undefined.

There is a growing body of evidence that implicates nitric oxide (NO) as a modulator of the adhesive interactions between cells that participate in acute and chronic inflammatory responses, i.e., leukocytes, platelets, and endothelial cells (13, 22, 23, 31). A role for NO in the pathobiology of I/R injury is supported by observations that NO generation is reduced in postischemic tissues (23) and that inhibition of NO biosynthesis, using various analogs of L-arginine (L-Arg), elicits most of the microvascular alterations (e.g., leukocyte adhesion, endothelial barrier dysfunction) observed in tissues exposed to I/R (22, 23). Furthermore, it has been shown that NO-donating compounds blunt the leukocyte recruitment and significantly protect against the microvascular dysfunction normally associated with I/R (23). More recently, we have reported that a low dose of the NO synthase inhibitor G-monomethyl-L-arginine (L-NMMA) exaggerates the hepatic leukostasis (leukocyte adherence in terminal hepatic venules and the sinusoidal plugging of leukocytes) and tissue hypoxia elicited by gut I/R, whereas treatment with either the NO donor diethylenetriamine/NO (DETA/NO) or L-Arg effectively blunts these responses to gut I/R (13). Because agents or conditions that favor the accumulation of NO afford a beneficial effect similar to that noted with interventions directed against P-selectin, the possibility exists that NO modulates the expression of P-selectin and the consequent recruitment of rolling leukocytes in venules of postischemic tissues. This possibility is supported by reports showing increased immunostaining of mesenteric venules exposed to NO synthase inhibitors (2) and a blunted expression of I/R-induced P-selectin in murine intestine pretreated with an NO donor (3). These studies, however, do not address whether the alterations in P-selectin expression observed after manipulation of tissue NO levels result in a corresponding quantitative change in the recruitment of rolling leukocytes.

The objectives of this study were to 1) determine whether gut I/R alters the expression of P-selectin in distant organs such as the liver and lung, 2) define the contribution of NO to the enhanced P-selectin expres-
NO modulates gut I/R-induced P-selectin expression. Changes in P-selectin expression profoundly affect the recruitment of rolling leukocytes. Our findings suggest that gut I/R leads to an increased expression of P-selectin in several organs, including small intestine, liver, and lung. In addition, our data indicate that NO modulates gut I/R-induced P-selectin expression in different organs and that the NO-mediated changes in P-selectin expression profoundly affect the recruitment of rolling leukocytes.

**MATERIALS AND METHODS**

**Animals.** All mice (8–12 wk old) used in this study were of a C57Bl background. Wild-type (control) mice were obtained from Jackson Laboratory (Bar Harbor, ME), whereas P-selectin-deficient mice (26) were obtained from Pharmacia-Upjohn (Kalamazoo, MI). The mutants were all developed in the 129 mouse strain, with eight backcrosses to C57Bl (12). The dual-radiolabeled antibody technique was used to verify the absence of P-selectin expression in unstimulated and cytokine-stimulated vascular beds (4). All mice were obtained at 4 wk of age and maintained on standard mouse chow until 18 h before the experiment.

**MAb.** The binding MAb used for the in vivo assessment of P-selectin expression was RB40.34, a rat IgG1 against mouse P-selectin (PharMingen, San Diego, CA) (4, 11, 29). P-23, a nonbinding murine IgG1 against human P-selectin (Pharmacia-Upjohn) was also used in the experimental protocols (25).

**Radiodination of MAb.** The binding MAb RB40.34 and the nonbinding MAb P-23 were radiolabeled with 125I and 131I (NEN, Boston, MA), respectively, using the iodogen method (11). This value, expressed as %ID, was converted to micrograms of MAb per gram of tissue by multiplying by the total injected binding MAb (µg), divided by 100.

**Surgical procedure for P-selectin expression experiments.** After atropine sulfate (0.04 mg/kg body wt ip) was administered, the mice were anesthetized with ketamine hydrochloride (150 mg/kg body wt im) and xylazine (7.5 mg/kg body wt im). The left jugular vein and the right carotid artery were cannulated with polyethylene tubing. A mixture of 10 µg of 125I-labeled anti-P-selectin MAb (125I-RB40.34) and an amount of 131I-labeled P-23 necessary to ensure a total 131I injected activity of 400,000–600,000 cpm was administered through the jugular vein cannula (total volume 200 µl). This dose was selected based on the results of pilot studies demonstrating optimum activity patterns and receptor saturation in the tissues examined under constitutive conditions (4). Previous studies employing blocking doses of unlabeled MAb RB40.34 indicate that its 125I-labeled counterpart accumulates in tissues via specific binding to its ligand (4).

In some mice, the superior mesenteric artery (SMA) was cannulated for 15 min, followed by 30 min of reperfusion. At 25 min after reperfusion, a mixture of the radiolabeled binding P-selectin MAb and the nonbinding MAb was administered intravenously. A sample of blood was obtained from the aortic cannula 5 min after MAb injection. Bicarbonate-buffered saline (BBS) was isovolemically exchanged by infusion of buffer through the jugular vein and simultaneous withdrawal of blood/buffer from the aorta after systemic heparinization (40 U/mouse). The vascular system was then copiously flushed with BBS through the aortic cannula after transaction of the inferior vena cava on the pleural aspect of the diaphragm. The following tissues were then harvested and weighed: lung, liver, mesentery, stomach, small intestine, large intestine, heart, and skeletal muscle. The experimental procedures described herein were performed according to criteria outlined in the National Research Council’s Guide for the Care and Use of Laboratory Animals (Washington, DC: Natl. Acad. Press, 1996).

**Calculation of P-selectin expression.** A 14800 Wizard 3 gamma counter (Wallac, Turku, Finland) with automatic correction for background activity and spill over was used to count 125I (binding MAb) and 131I (nonbinding MAb) activities in each organ or tissue and in a 50-µl plasma sample. A 4-µl aliquot of the preinjection mixture of radiolabeled MAb was assayed to determine total injected activity of each labeled MAb. The radioactivities remaining in the tube used to mix the MAb, the syringe used to inject the mixture, and the jugular vein catheter were subtracted from the total calculated injected activity. P-selectin expression was determined by subtracting the accumulated activity of the nonbinding MAb (131I-labeled P-23) from that of the binding MAb activity (125I-RB40.34) and was expressed as the percentage of the injected dose (%D) per gram of tissue. The formula used to calculate P-selectin expression was as follows

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\text{P-selectin expression} = \left( \frac{\%D_{125I}}{\%D_{131I}} \right) \times \frac{\%D_{125I}\text{-plasma}}{\%D_{131I}\text{-plasma}}
\]

This formula was modified from the original method to correct the tissue accumulation of nonbinding MAb for the relative plasma levels of both binding and nonbinding MAb (11). This value, expressed as %D, was converted to micrograms of MAb per gram of tissue by multiplying by the total injected binding MAb (µg), divided by 100.

**Surgical procedure for intravital microscopic study.** After atropine sulfate (0.04 mg/kg body wt ip) was administered, the mice were anesthetized with ketamine hydrochloride (150 mg/kg body wt im) and xylazine (7.5 mg/kg body wt im). The right carotid artery was cannulated, and systemic arterial pressure was measured with a Statham P23A pressure transducer (Gould, Oxnard, CA) connected to the carotid artery cannula. Systemic blood pressure and heart rate were continuously recorded with a physiological recorder (Grass Instruments, Quincy, MA). The left jugular vein was also cannulated for drug administration. After laparotomy, the SMA was occluded with a microvascular clip for 0 (sham) or 15 min. After the ischemic period, the clip was gently removed.

**Intravital microscopy.** Immediately after the clip was removed, the mouse was placed on a microscope stage. A lobe of liver was observed with an inverted intravital microscope (TMD-2S, Diaphot, Nikon, Tokyo, Japan) assisted by a silicon-
Although this low dose of L-NMMA does not completely inhibit NO synthase, it profoundly affects the responses of the hepatic microcirculation to bacterial endotoxin. The re-administration of L-NMMA (0.5 mg/kg) per se neither induced P-selectin expression in any tissues nor increased the number of rolling leukocytes in the terminal hepatic venules (THV). Gut I/R elicited an increased P-selectin expression in lung, liver, mesentery, small intestine, and large intestine, but it did not affect this adhesion molecule in either the heart or skeletal muscle.

Figure 1 summarizes the changes in P-selectin expression in the liver after exposure of the gut to ischemia and 30 min reperfusion. Also shown are the effects of an NO synthase inhibitor, L-NMMA (Fig. 1A), L-Arg, and D-Arg (Fig. 1B) on P-selectin expression in liver after 15 min of gut ischemia and 30 min of reperfusion (I/R). Also shown are data for coadministration of L-NMMA with either L-arginine (L-Arg) or D-arginine (D-Arg). DETA, diethylenetriamine; MAB, monoclonal antibodies. Data are means ± SE for 5 animals in each experimental group. *P < 0.05 vs. control; #P < 0.05 vs. I/R (untreated) group.
an NO donor, DETA/NO (Fig. 1B), on these responses. 
Gut I/R induced a significant (3.7-fold) increase of the 
expression of P-selectin in the liver. The gut I/R-
induced increase in the expression of hepatic P-selectin 
was enhanced (~90%) by the administration of 
L-NMMA, whereas treatment with either L-Arg or 
DETA/NO, but not with D-Arg or DETA, completely 
blocked the gut I/R-induced response. The exacerbation 
of gut I/R-induced hepatic P-selectin expression by 
L-NMMA was prevented by coadministration of L-Arg, 
but not D-Arg.

Figure 2 illustrates the changes in P-selectin expres-
sion in the gastrointestinal tract (mesentery, stomach, 
small intestine, and large intestine) after exposure of 
the gut to ischemia and 30-min reperfusion. Also shown 
are the effects of L-NMMA (Fig. 2A), L-Arg, and 
DETA/NO (Fig. 2B) on these responses. Gut I/R in-
duced two- to threefold increases in the expression of 
P-selectin in the mesentery, small intestine, and large 
intestine but did not alter expression in the stomach.

L-NMMA enhanced the gut I/R-induced increase in 
P-selectin expression in mesentery (90%), small intes-
tine (150%), and large intestine (170%). Coadministra-
tion of L-Arg, but not D-Arg, prevented the L-NMMA-
induced exacerbation of the gut I/R response. Treatment 
with either L-Arg or DETA/NO prevented the gut 
I/R-induced increase in P-selectin expression in mesen-
tery, small intestine, and large intestine.

Figure 3 shows that gut I/R elicited a significant 
increase (doubling) in the expression of P-selectin in 
the lung. Although L-NMMA did not exacerbate the 
lung response (Fig. 3A) to gut I/R, treatment with 
either L-Arg or DETA/NO (Fig. 3B) did prevent the gut 
I/R-induced rise in P-selectin expression.

Figure 4 illustrates the effect of gut I/R on the flux of 
rolling leukocytes in THV. The flux of rolling leukocytes 
after gut I/R was 2.8 times the value obtained in THV 
under basal conditions. L-NMMA treatment further 
increased the flux of rolling leukocytes by 53%, a 
response that was prevented by coadministration of 
L-Arg but not D-Arg (Fig. 4A). The enhanced leukocyte
rolling in THV elicited by gut I/R (with or without L-NMMA treatment) was not observed in P-selectin-deficient mice. Treatment with either L-Arg (but not D-Arg) or DETA/NO (but not DETA) largely abolished the recruitment of rolling leukocytes in THV that was normally elicited by gut I/R.

Gut I/R resulted in a significant reduction in leukocyte rolling velocity in THV from 66.6 ± 3.5 µm/s (control) to 46.6 ± 4.2 µm/s (after gut I/R). This response was not altered by L-NMMA (45.6 ± 4.5 µm/s), L-Arg (49.1 ± 4.7 µm/s), or DETA/NO (53.4 ± 3.6 µm/s).

**DISCUSSION**

Depletion and/or inactivation of NO has been implicated as a key event in eliciting the acute inflammatory response observed in a variety of tissues exposed to I/R, with corroborating evidence provided by reports describing a blunted inflammatory response when tissue NO levels are restored using NO-donating compounds (13, 19, 23, 32). Previously published work has demonstrated that reperfusion of the ischemic small intestine elicits acute inflammatory responses both in the intestinal (8, 23) and in distant organs such as liver (12–14, 33) and lung (1, 33). In the liver, this response is characterized by leukocyte plugging of sinusoids, leukocyte adherence in THV, a reduction in the number of perfused sinusoids, hepatocellular hypoxia, and leakage of enzymes (alanine aminotransferase) from hepatocytes (8, 10, 14, 33). Most of the microvascular and parenchymal cell alterations observed in the liver after gut I/R are exacerbated by NO synthase inhibitors and attenuated by NO-generating agents (13). The dependence of this hepatic microvascular dysfunction and hepatocellular hypoxia/injury on leukocyte sequestration is evidenced by the improved sinusoidal perfusion and blunted hepatocellular hypoxia/injury responses to gut I/R observed in mice that are genetically deficient in one of a variety of adhesion glycoproteins, including intercellular adhesion molecule 1, P-selectin, and CD11/CD18 (12). Although there is substantial evidence that implicates NO as a modulator of I/R-induced leukocyte recruitment (13, 19, 23, 32), relatively little is known about how NO exerts this beneficial action on leukocyte-endothelial cell adhesion. Hence, in this study we examined the possibility that NO blunts gut I/R-induced leukocyte rolling in hepatic venules by interfering with the endothelial expression of P-selectin.

The present study has resulted in several novel observations that improve our understanding of the mechanism that accounts for the recruitment of leukocytes into the hepatic microvasculature after gut I/R: 1) P-selectin expression is rapidly and significantly increased in the liver, gastrointestinal tract, and lung after gut I/R, 2) this upregulation of P-selectin expression in the liver is associated with a corresponding recruitment of rolling leukocytes in THV, and 3) both the upregulation of P-selectin and recruitment of rolling leukocytes in the liver after gut I/R are exacerbated by NO synthase inhibition and blunted by interventions (NO donors, L-Arg supplementation) that restore tissue NO levels. These findings, coupled with our observation that P-selectin-deficient mice do not exhibit leukocyte rolling after gut I/R (with or without L-NMMA), support the view that P-selectin-dependent mechanisms largely account for the recruitment of rolling leukocytes in the liver after gut I/R and that this component of the inflammatory process may be controlled by NO.

It is now recognized that gut I/R not only causes local microvascular and mucosal degenerative changes but also elicits an inflammatory response and tissue injury in downstream organs, such as liver (12–14, 33) and lung (1, 33). A role for P-selectin in these distant organ responses to gut I/R has been implicated on the basis of reports demonstrating diminished distant organ injury in animals that either receive P-selectin blocking antibodies (1) or are genetically deficient in P-selectin (12). It has previously been proposed that P-selectin-directed interventions (e.g., MAb) protect against distant organ injury after gut I/R because they attenuate injury to the gut per se, which consequently results in a blunted injury response in the downstream organ (1, 12). However, our observation that gut I/R elicits an
increased P-selectin expression in both the liver and lung would favor the possibility that this adhesion molecule directly contributes to the gut I/R-induced injury process at these distant sites. In this regard, it is noteworthy that gut I/R induced a more pronounced upregulation of P-selectin in the liver (3.7-fold) compared with lung (twofold). This observation suggests that the chemicals released from the gut that promote P-selectin expression reach the liver at a higher concentration than when they reach the lung. The fact that P-selectin expression increased by a greater extent in the liver than the small intestine (doubling) would argue against ischemia per se as a stimulus for the greater adhesion molecule upregulation in liver than lung.

An interesting finding in the present study is that a low dose of the NO synthase inhibitor L-NMMA (28) enhanced both the flux of rolling leukocytes and P-selectin expression that were normally elicited in the liver by gut I/R. The fact that this recruitment of additional rolling leukocytes was not observed in P-selectin-deficient mice strongly indicates a cause-effect relationship between the increased P-selectin expression and recruitment of rolling leukocytes in the liver. These observations also support the view that although gut I/R may reduce NO bioavailability in the liver, there remains a substantial level of NO production that can be inhibited by the low dose of L-NMMA. Furthermore, these findings are consistent with the notion that the NO generated by vascular endothelial cells serves to scavenge superoxide anions (15) produced at an accelerated level after I/R (8). It has been shown that both I/R-induced (17, 21, 34) and L-NAME-induced (2) P-selectin-dependent microvascular alterations are blunted by superoxide dismutase. I:nasmuch as superoxide is known to elicit an increased P-selectin expression on endothelial cells (30), one might expect that inhibition of NO production during periods of accelerated superoxide production (e.g., I/R) would result in the observed increase in P-selectin expression and P-selectin-dependent leukocyte rolling.

Two strategies were used to maintain or elevate the tissue level of NO after gut I/R, supplementation with L-Arg (the substrate for NO synthase) or administration of the NO-donating agent DETANO. These interventions (but not d-Arg or DETAN) effectively prevented both the gut I/R-induced leukocyte rolling in THV and the P-selectin upregulation observed in the liver, gastrointestinal tract, and lung. Whether the NO-replenishing agents prevent the P-selectin upregulation in organs distant to the gut by acting directly on these vascular beds or by preventing the production and/or release of inflammatory substances (e.g., histamine) from the gut that can enhance P-selectin expression remains unclear. However, our findings with the NO-replenishing interventions are consistent with the L-NMMA data in that they invoke an inhibitory action of NO on leukocyte rolling mediated via the regulation of P-selectin expression. Although an inhibitory effect of NO donors on leukocyte-endothelial cell adhesion (5–7, 9, 13, 23) and P-selectin expression (3) has been described in different reports, this study provides evidence for a cause-effect relationship between the two variables. Nonetheless, there are published data that do not support a major role for P-selectin in I/R-induced leukocyte-endothelial cell adhesion. For example, Kubes et al. (18) reported that NO donors do not prevent P-selectin-dependent leukocyte rolling in postischemic venules of cat mesentery (18). In addition, although P-selectin-specific MAb do not affect I/R-induced leukocyte adhesion in rat mesenteric venules (20), NO-donating compounds exert a profound inhibitory effect in the same model system (23). An explanation for the inconsistent findings regarding the effectiveness of (and linkage between) P-selectin MAb and NO-donating agents in models of I/R injury is not readily available; however, the outcome of these studies is likely to be influenced by the nature and severity of the ischemic insult, the species and tissue examined, the time of agent administration (ischemia vs. reperfusion), and the contribution of auxiliary cells (mast cells, platelets, macrophages) to the inflammatory response.

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