Role of nitric oxide in gut ischemia-reperfusion-induced hepatic microvascular dysfunction

YOSHINORI HORIE,1 ROBERT WOLF,2 AND D. NEIL GRANGER1

Departments of 1Molecular and Cellular Physiology and 2Medicine, Center of Excellence in Arthritis and Rheumatism, Louisiana State University Medical Center, Shreveport, Louisiana 71130

Horie, Yoshinori, Robert Wolf, and D. Neil Granger. Role of nitric oxide in gut ischemia-reperfusion-induced hepatic microvascular dysfunction. Am. J. Physiol. 273 (Gastrointest. Liver Physiol. 36): G1007–G1013, 1997.—The overall objective of this study was to assess the contribution of an altered bioavailability of nitric oxide (NO) to the leukocyte adhesion and hypoxic stress elicited in the liver by gut ischemia-reperfusion (I/R). The accumulation of leukocytes, number of nonperfused sinusoids (NPS), and NADH autofluorescence were monitored (by intravital microscopy) in mouse liver after 15 min of superior mesenteric artery occlusion and 60 min of reperfusion. Leukostasis, NPS, and NADH autofluorescence (indicating hypoxia) were all increased in the liver at 60 min after gut I/R. The NO synthase inhibitor N’-monomethyl-L-arginine (L-NMMA) exaggerated the liver leukostasis elicited by gut I/R, responses that were prevented by coadministration of L-arginine. The NO donor diethylenetriamine-NO (DETA-NO) and L-arginine were both effective in attenuating the gut I/R-induced leukostasis and increased NADH autofluorescence, whereas neither DETA nor L-arginine exerted a protective action. These findings indicate that NO is an important determinant of the liver leukostasis, impaired sinusoidal perfusion, and tissue hypoxia elicited by gut I/R.

leukocyte-endothelial cell adhesion; nitric oxide synthase; tissue hypoxia

IT IS WELL RECOGNIZED THAT nitric oxide (NO) is an important modulator of tissue blood flow, arterial pressure, neurotransmission, and immune cell function (25). There is also growing evidence that implicates NO as a modulator of the adhesive interactions among leukocytes, platelets, and endothelial cells (18, 19, 25, 28). These NO-dependent cell-cell interactions have been demonstrated in tissues exposed to ischemia-reperfusion (I/R), an injury process in which leukocyte-endothelial cell adhesion plays a critical role. A role for NO in the pathobiology of I/R injury is supported by observations that NO generation is reduced in postischemic tissues (19) 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 (7, 19). Furthermore, it has been shown that NO-donating compounds provide significant protection against the microvascular dysfunction that is normally associated with I/R (19).

Several cell types are known to produce NO, including endothelial cells (25), macrophages (25), neurons (6), and neutrophils (25). The liver, for example, has the capacity to generate NO from three different resident cell populations, i.e., Kupffer cells (1), hepatocytes (25), and endothelial cells (25). There are some observations generated from in vitro and in vivo model systems that implicate NO as a cytotoxic mediator in the liver. NO appears to inhibit protein synthesis and mitochondrial respiration in isolated hepatocytes (5, 32). Furthermore, there is evidence from isolated perfused rat livers indicating that NO synthase (NOS) inhibitors attenuate lipopolysaccharide (LPS)-induced mitochondrial dysfunction (17). There are also studies that invoke a cytoprotective effect of NO in the liver. For example, it has been reported that cytokine-induced stimulation of endogenous NO production decreases hepatocellular injury elicited by acetaminophen (16). In addition, endotoxemic mice exhibit an exaggerated hepatic microcirculatory dysfunction (leukosequestration and thrombus formation) and more severe liver damage after treatment with NOS inhibitors (1, 8, 27).

A similar inconsistency of responses to NO inhibition has been observed in tissues exposed to I/R. Some studies (7, 19) invoke a cytoprotective effect of NO in I/R injury that appears to be mediated through the attenuation of leukocyte recruitment, whereas the findings of other studies (3, 24) suggest that NO actually contributes to I/R-induced injury in different tissues, including the liver (22). It has been suggested (13) that inhibition of NO synthesis in vivo does not affect reperfusion injury to the liver unless the animals are pretreated with endotoxin (37), which presumably primes the Kupffer cells for accelerated production of toxic NO metabolites.

We have recently developed a leukocyte-dependent model of hepatocellular dysfunction elicited by gut I/R (10). This murine model allows for an in vivo assessment of the effects of I/R on leukocyte sequestration in sinusoids of different regions of the liver lobule, leukocyte adherence in postsinusoidal venules, the number of perfused sinusoids, and NADH autofluorescence (an index of tissue hypoxia). Using this model, we have examined how an altered bioavailability of NO, induced either by inhibition of NOS or supplementation with exogenous NO, affects the leukocyte adhesion, sinusoidal perfusion, and oxidative stress elicited in the liver by gut I/R.

MATERIALS AND METHODS

Animals. All mice (n = 95; 8–12 wk in age) used in this study were of a C57Bl background and were obtained from Harlan-Sprague Dawley. The mice were fed a standard mouse chow and fasted for 18 h before the experiment.

Surgical procedure. After administration of atropine sulfate (0.04 mg/kg body wt ip), 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 superior mesenteric artery (SMA) was occluded with a microvascular clip for 0 (sham) or 15 min. After the ischemic period, the clip was gently removed. Estimates of blood flow using laser-Doppler flowmetry indicate that ligation of the SMA results in an ~70% reduction in blood flow in mouse liver.

Intravital microscopy. Immediately after removing the clip, 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-intensified target (SIT) camera (C-2400-08, Hamamatsu Photonics, Shizuka, Japan). The liver was placed on an adjustable Plexiglas microscope stage with a nonfluorescent coverslip that allowed for observation of a 2-cm² segment of tissue. The liver was carefully positioned to minimize the influence of respiratory movements. The liver surface was moistened and covered with cotton gauze soaked with saline. Images of the microcirculation near the surface of the liver were observed through a ×40 fluorescent objective lens (Fluor 400/0.85, Nikon). The microfluorographs were recorded on videotape using a videocassette recorder (NV8950, Panasonic, Tokyo, Japan). A video time-date generator (WJ 810, Panasonic) projected the stopwatch function onto the monitor.

Analysis of leukocyte accumulation and sinusoidal perfusion in liver microcirculation. Leukocytes were labeled in vivo with rhodamine 6G (2 mg in 5 ml of 0.9% saline), using a previously described method (10). It has been shown that rhodamine 6G selectively stains white blood cells and platelets, but not endothelial cells (21). Thus the fluorochrome allows for differentiation between adherent leukocytes and endothelial cells. Rhodamine 6G (0.4 ml/100 g body wt) was injected before gut reperfusion with subsequent injections every 30 min. Rhodamine 6G-associated fluorescence was visualized by epi-illumination at 510–560 nm, using a 590-nm emission filter. The number of stationary leukocytes was determined off-line during playback of videotape images. A leukocyte was considered stationary within the microcirculation (sinusoids) if it remained motionless for more than 10 s. The sinusoid was considered perfused if the labeled white blood cells or platelets were observed moving through it. The percentage of nonperfused sinusoids was calculated as the ratio of the number of nonperfused sinusoids to the total number of sinusoids per viewing field. Stationary leukocytes were quantified in both the midzonal and pericentral regions of the liver lobule and expressed as the number per field of view (3.3 x 10³ μm²).

Analysis of autofluorescence of pyridine nucleotide. Autofluorescence of pyridine nucleotide (NADH) was measured as an indicator of mitochondrial O2 consumption using the same intravital microscopy system. Autofluorescence of NADH has been used as a measure of the redox state in various tissues (10, 11, 33, 34, 36). However, a study by Sueyama et al. (33) suggests that ultravioleto-elicited intrahepatic autofluorescence also reflects vitamin A. Thus, to eliminate intrinsic vitamin A autofluorescence, which is mainly found in fat droplets of Ito cells, we exposed the liver surface to ultraviolet epi-illumination for 20 s; this period was enough to abolish vitamin A autofluorescence because of its rapid photobleaching properties. NADH autofluorescence was visualized with a SIT camera by epi-illumination at 300–360 nm, using a 450-nm emission filter. Autofluorescence of NADH in both pericentral and midzonal regions of the liver was recorded on videotape immediately after quantification of leukocyte accumulation and measured with a computer-assisted digital-imaging processor (NIH Image 1.35 on a Macintosh computer). The temporal changes in autofluorescence intensity of NADH \( \frac{d[I_{\text{NADH}}(x,y,t)]}{dt} \) (count/pixel) were calculated according to the following equation

\[
\frac{d[I_{\text{NADH}}(x,y,t)]}{dt} = \frac{I_{\text{NADH}}(x,y,t) - I_{BG}(x,y,t)[I_{\text{NADH}}(x,y,15) - I_{BG}(x,y,15)]}{100}
\]

where \( I_{BG}(x,y,t) \) is the background intensity, which was measured in the center of the terminal hepatic venules (THV).

Preliminary experiments. Some experiments were performed to determine how the lower and higher ends of NG-monomethyl-L-arginine (L-NMMA; Sigma Chemical, St. Louis, MO) doses previously used to inhibit NOS (27) affect leukocyte accumulation, NADH autofluorescence, and the number of nonperfused sinusoids in murine liver. In these studies, the aforementioned variables were measured every 15 min for 90 min after administration of saline (control) or L-NMMA (0.5 or 30 mg/kg).

Experimental protocols. The SMA was occluded with a microvascular clip for 0 (sham) or 15 min. After the ischemic period, the clip was gently removed. Leukocyte accumulation, autofluorescence of NADH, and the number of nonperfused sinusoids were measured 15 min after reperfusion and every 15 min for 45 min thereafter, that is 60 min after reperfusion. In one series of experiments, the mice were divided into three different groups that were given the following intravenous treatments (15 min before ischemia): 1) L-NMMA (0.5 mg/kg), 2) L-NMMA (0.5 mg/kg) and L-Arg (2.5 mg/kg), or 3) L-NMMA (0.5 mg/kg) and D-arginine (D-Arg; 2.5 mg/kg). Four additional groups were given the following treatments (also 15 min before ischemia): 4) L-Arg (0.5 mg·kg⁻¹·h⁻¹ iv), 5) D-Arg (0.5 mg·kg⁻¹·h⁻¹ iv), 6) diehydrotiamine (DETA)-NO, an NO donor with a 20-h half-life (51.7 mg/kg ip; 2 h before control measurements) (12, 26), and 7) DETA (32.7 mg/kg ip) (Aldrich, Milwaukee, WI).

In 20 additional mice, the same gut I/R protocol (as described above) was employed; however, plasma alanine aminotransferase (ALT) measurements were obtained at 6 h after reperfusion. These experiments were performed in control mice (sham gut I/R), after gut I/R (no treatment), gut I/R plus DETA, and gut I/R plus DETA-NO, with five animals in each group.

Enzyme assay. Blood samples were collected from the carotid artery after obtaining the 60-min or 6-h reperfusion measurements. Plasma ALT activity was determined from these samples using a spectrophotometric assay obtained as a commercial kit (Sigma Chemical).

Statistics. The data were analyzed using standard statistical analyses, i.e., one-way analysis of variance and Scheffé’s (post hoc) test. All values are reported as means ± SE, with five to six mice per group. Statistical significance was set at \( P < 0.05 \).

RESULTS

Table 1 compares the leukostasis, sinusoidal perfusion, and NADH autofluorescence responses (at 90 min) after administration of a low (0.5 mg/kg) and high (30 mg/kg) dose of L-NMMA, which were selected from a previous study by Nishida et al. (27). The high L-NMMA dose (30 mg/kg) resulted in enhanced leukostasis, an increase in the percentage of nonperfused sinusoids, and an elevated NADH autofluorescence. The lower dose of L-NMMA (0.5 mg/kg) did not elicit significant
changes in any of the measured variables, consistent with a previous report (27).

Figure 1 summarizes the changes in leukocyte accumulation that occur in sinusoids of the midzonal and pericentral regions of the liver lobule, within the THV, and in the entire liver lobule (sinusoids and THV; Fig. 1B) after exposure of the gut to ischemia and 60 min reperfusion. Also shown are the effects of L-NMMA on these responses. L-NMMA aggravated the leukostasis elicited by gut I/R in the pericentral region (5.4 ± 0.55 I/R vs. 7.8 ± 0.30 I/R plus L-NMMA). The NOS inhibitor also increased the total number of stationary leukocytes (18.0 ± 1.7 I/R vs. 24.1 ± 1.8 I/R plus L-NMMA). This enhancement of leukostasis was prevented by coadministration of L-Arg (4.6 ± 0.42, pericentral; 16.5 ± 0.84 total) but not of D-Arg.

Figure 2 depicts how L-Arg and the NO donor DETA-NO affect the recruitment of stationary leukocytes, nonperfused sinusoids, and NADH autofluorescence in control mice.

Table 1. Effects of L-NMMA on stationary leukocytes, nonperfused sinusoids, and NADH autofluorescence in control mice

<table>
<thead>
<tr>
<th>Stationary Leukocytes, per field</th>
<th>NADH Autofluorescence, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midzonal</td>
<td>Pericentral</td>
</tr>
<tr>
<td>Control</td>
<td>2.0 ± 0.31</td>
</tr>
<tr>
<td>L-NMMA (0.5 mg/kg)</td>
<td>2.8 ± 0.20</td>
</tr>
<tr>
<td>L-NMMA (30 mg/kg)</td>
<td>5.0 ± 0.31*</td>
</tr>
</tbody>
</table>

Values are means ± SE. L-NMMA, N⁰-monomethyl-L-arginine; NPS, nonperfused sinusoids; THV, terminal hepatic venules. *P < 0.05 vs. control.
cytes in the liver after gut I/R. L-Arg reduced the leukocyte sequestration elicited by gut I/R in the pericentral region (5.4 ± 0.55 I/R vs. 3.2 ± 0.36 I/R plus L-Arg), and DETA-NO reduced leukostasis in the pericentral region (3.1 ± 0.14) and in the THV (7.4 ± 1.5 I/R vs. 2.2 ± 0.18 I/R plus L-Arg). L-Arg and DETA-NO each reduced the total number of stationary leukocytes elicited by gut I/R. D-Arg and DETA did not significantly alter the leukocyte recruitment response elicited by gut I/R in any vascular region of the liver.

Figure 3 shows the changes in the percentage of nonperfused sinusoids elicited by gut I/R as well as the effects of L-NMMA (Fig. 3A) or L-Arg and DETA-NO (Fig. 3B) on these responses. Gut I/R resulted in an increase in the percentage of nonperfused sinusoids. This response was unaffected by L-NMMA (with or without L- or D-Arg). However, both L-Arg and DETA-NO were very effective in reducing the percentage of nonperfused sinusoids elicited by gut I/R (33.0 ± 2.9 I/R vs. 19.4 ± 2.6 I/R plus L-Arg, 19.1 ± 1.8 I/R plus DETA-NO). Neither D-Arg nor DETA altered the sinusoidal perfusion responses to gut I/R.

Figure 4 summarizes the changes in NADH autofluorescence in the midzonal and pericentral regions of the liver after gut I/R, and how L-NMMA, L-Arg, and DETA-NO affect these responses. The intensity of NADH autofluorescence was significantly increased with gut I/R, with more substantive increases noted in the pericentral region. Although L-NMMA (with or without L- or D-Arg) treatment did not modify the NADH autofluorescence response to gut I/R, both L-Arg and DETA-NO were effective in blunting the NADH autofluorescence response in the pericentral region, but not in the midzonal region. D-Arg and DETA did not affect the NADH autofluorescence response to gut I/R in either region of the liver.

Figure 5 shows the plasma ALT changes elicited by gut I/R and how L-NMMA, L-Arg, and DETA-NO affect the I/R-induced changes. L-NMMA enhanced the gut I/R-induced increase of plasma ALT, a response that was prevented by coadministration of L-Arg but not of D-Arg. In the presence of DETA-NO, gut I/R did not elicit a significant increase in plasma ALT. Neither
L-Arg, D-Arg, nor DETA alone affected the gut I/R-induced elevation in plasma ALT.

Table 2 summarizes the changes in plasma ALT activity observed at 6 h after gut I/R and the effects of DETA-NO on this response. A more substantial increase in plasma ALT activity occurred in animals with 6 h of reperfusion compared with those with 1 h of reperfusion. Furthermore, DETA-NO, but not DETA, significantly attenuated the 6-h elevation in plasma ALT.

DISCUSSION

Previously published work has demonstrated that reperfusion of the ischemic small intestine elicits an acute inflammatory response both in the intestine and in distant organs, such as the liver (10, 11) and the lung (2). In the liver, this response is characterized by leukocyte plugging of sinusoids, leukocyte adherence in postcapillary venules, a reduction in the number of perfused sinusoids, hepatocellular hypoxia, and leakage of enzymes (ALT) from hepatocytes (9, 10, 11, 31). 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. These responses are observed in mice that are genetically deficient in one of a variety of adhesion glycoproteins, including intercellular adhesion molecule 1 (ICAM-1), P-selectin, and CD11/CD18 (10). Because depletion and/or inactivation of NO has been implicated as a key event in the recruitment of leukocytes in tissues exposed to I/R (15, 23, 30), we chose to determine whether manipulation of hepatic NO levels (either by NOS inhibition with L-NMMA, NOS stimulation with L-Arg, or administration of an NO donor) alters the leukostasis and leukocyte-dependent cell injury that are normally observed in the liver after gut I/R.

The results of our study provide three lines of evidence that support a potential role for NO as an endogenous agent that protects the liver from the deleterious effects of gut I/R:

1. NOS inhibition with a high dose of L-NMMA elicits inflammatory and microvascular responses that are nearly identical to those observed in the liver after gut I/R (Table 1),
2. low-dose treatment with L-NMMA results in an exaggerated leukostasis and cellular injury in the liver after gut I/R, and
3. increased delivery and/or generation of NO in the liver via an NO donor or L-Arg (substrate for NOS) attenuates the inflammatory responses and microvascular dysfunction elicited in the liver by gut I/R. The fact that a high L-NMMA dose, which should yield complete inhibition of NOS (35), elicits microvascular responses in the liver that are consistent with inflammation is in keeping with previously published observations on other tissues (7, 15). NOS inhibition, induced with one of a variety of L-Arg analogs (L-NMMA, N\textsuperscript{G}-nitro-L-arginine methyl ester; N\textsuperscript{G},N\textsuperscript{N}-dimethyl-L-arginine), has been shown to cause leukocyte adherence and emigration, platelet-leukocyte aggregation, enhanced oxidant production, and albumin leakage in postcapillary venules as well as mast cell degranulation in the surrounding interstitial compartment (7, 18, 20). These changes closely mimic the microvascular responses that are observed in tissues exposed to I/R. The latter observation, coupled to reports describing a reduction in tissue NOS activity (14) and diminished NO production (7, 19), suggests that diminished NO levels in postischemic tissues lead to the formation of...
the inflammatory mediators that result in leukocyte activation and recruitment. The isof orm of NOS that contributes to the gut I/R-induced inflammatory responses in the liver remains unclear. However, because several hours are required for induction of inducible NOS (25), constitutive (endothelial) NOS appears to be a more likely candidate for the gut I/R-induced inflammatory responses observed in our model.

Our observation that low-dose treatment with L-NMMA results in an exaggerated leukostasis and cellular injury in the liver after gut I/R suggests that significant NOS activity is retained after I/R. Previously published work (19) on the mesenteric microvasculature indicates that the inflammatory responses to I/R are not exacerbated by prior administration of NOS inhibitors, suggesting that I/R per se nearly completely inhibits NOS activity in the mesentery. The results of the present study may, however, be explained by the fact that the liver is only partially ischemic during SMA occlusion; hence the stimulus for NOS inhibition in the liver may not be as severe.

Two strategies were used to maintain or elevate the tissue level of NO after gut I/R, supplementation with L-Arg (the substrate for NOS) or administration of the NO donor compound DETANO. These interventions, but not D-Arg or DETA, proved to be effective in blunting the leukostasis, sinusoidal obstruction, and tissue hypoxia induced by gut I/R. These observations are consistent with the view that NO is an effective inhibitor of leukocyte-endothelial cell adhesion (7, 15, 19, 20) and that leukocytes account for the inadequate perfusion of sinusoids and tissue hypoxia (increased NADH autofluorescence) that result from gut I/R. In the present study we observed that gut I/R-induced leukostasis in the midzonal region of the liver lobule was not attenuated by either DETANO or L-Arg. A similar absence of an inhibitory response in the midzonal region was noted in mice that are genetically deficient in either CD11/CD18, ICAM-1, or P-selectin, compared with their wild-type controls (10). This consistency of the zone-dependent responses between the two studies favors a role for leukocytes and suggests that the leukocyte accumulation elicited by gut I/R in the midzonal region is likely caused by physical hindrance or plugging.

As shown in Table 2, 6 h of reperfusion after SMA occlusion caused a more substantial increase of ALT levels. This finding indicates that gut I/R can cause substantial hepatocellular injury. We also noted that an NO donor (DETA-NO) is capable of attenuating both the initial ALT leakage from hepatocytes that occurs at 1 h after reperfusion as well as the later (6 h), more substantial rise in plasma ALT level.

An interesting yet unresolved issue related to this work concerns whether the gut, liver, or both are the target tissue on which the protective effects of L-Arg and DETANO are conferred in our model of gut I/R-induced hepatic microvascular dysfunction. DETANO and L-Arg, which were administered systemically, may act directly on the liver microvasculature to prevent the leukostasis and subsequent leukocyte-dependent events and/or may act at the level of the gut to prevent the release of inflammatory agents that activate the leukocytes that either lodge in downstream sinusoids or adhere in THV. The latter possibility appears tenable inasmuch as both NO donors (7, 19) and L-Arg have been shown to protect the small bowel against the injurious effects of I/R. A similar case may be made for the former mechanism (direct effect on liver) since there are reports (29) demonstrating a protective effect of L-Arg in liver I/R. Further work is needed to define the contribution of the gut to the protective effects afforded by NO-generating strategies in this model of liver injury.

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

Address for reprint requests: D. N. Granger, Dept. of Physiology, Louisiana State University Medical Center, 1501 Kings Highway, Shreveport, LA 71130-3932.

Received 17 March 1997; accepted in final form 15 July 1997.

REFERENCES


