NAD(P)H oxidase contributes to the progression of remote hepatic parenchymal injury and endothelial dysfunction, but not microvascular perfusion deficits

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Am J Physiol Gastrointest Liver Physiol 290: G1025–G1032, 2006. First published December 8, 2005; doi:10.1152/ajpgi.00246.2005.—Oxidative stress occurs in remote liver injury, but the origin of the oxidant generation has yet to be thoroughly delineated. Some reports suggest that the source of the distant oxidative stress originates from the site of initial insult [i.e., xanthine oxidase (XO)]; however, it could also be derived from sources such as phagocytic and/or vascular NAD(P)H oxidase (Nox) enzymes. With a murine model of bilateral hindlimb ischemia–reperfusion, we describe here a mechanism for Nox-dependent oxidant production that contributes, at least in part, to remote hepatic parenchymal injury and sinusoidal endothelial cell (SEC) dysfunction. To determine whether Nox enzymes were the source of oxidants, mice were treated immediately after the onset of hindlimb ischemia with specific inhibitors to XO (50 mg/kg ip allopurinol) or Nox (10 mg/kg ip gp91ds-tat and 3 mg/kg ip apocynin). After 1 h of ischemia, hindlimbs were reperfused for either 3 or 6 h. Inhibition of XO failed to provide any improvement in parenchymal injury, SEC dysfunction, neutrophil accumulation, or microvascular dysfunction. In contrast, the inhibition of Nox enzymes prevented the progression (6 h) of parenchymal injury, significantly protected against SEC dysfunction, and completely prevented signs of neutrophil-derived oxidant stress. At the same time, however, inhibition of Nox failed to prevent against the early parenchymal injury and microvascular dysfunction at 3 h of reperfusion. These data confirm that microvascular perfusion deficits are not essential for the pathogenesis of remote hepatic parenchymal injury. The data also suggest that Nox enzymes, not XO, are involved in the progression of compromised hepatic parenchymal and endothelial integrity during a systemic inflammatory response.

xanthine oxidase; intravital microscopy; inflammation

PARASITEMIA, endotoxemia, trauma, severe burns, and pancreatitis can result in a systemic inflammatory response that could progress to multiple-organ dysfunction and ultimately organ failure (9, 34). Previously, it has been shown that hindlimb ischemia–reperfusion (HIR) results in systemic inflammation (46, 52, 53). It has also been shown that remote liver injury occurs early in this model, with progressive increases over time (5–7, 46, 53). In addition to elevated hepatic parenchymal injury, there is well-documented evidence of microvascular dysfunction with perfusion deficits and increased leukocyte-endothelial cell interactions (51). After HIR, the presence of a hepatic oxidative stress has been established (46), yet the origin of this stress remains to be elucidated completely. Therefore, we investigated two possible sources of oxidants, xanthine oxidase (XO) and NAD(P)H oxidase (Nox), to ascertain their contributions to the oxidant-mediated hepatic injury that occurs in the setting of a systemic inflammatory response.

Xanthine oxidoreductase, a molybdenum-pterin protein, is the rate-limiting enzyme catalyzing the oxidation of hypoxanthine to xanthine and urate (21). Under the oxidative and proteolytic conditions of HIR, the dehydrogenase form of xanthine oxidoreductase (XDH) converts to the oxidase form (XO). Rather than using NADH as the terminal electron acceptor like XDH, XO utilizes O2- and yields O2- and H2O2 (37). Numerous investigations have suggested that XO is released into the circulation during severe inflammatory states and binds to glycosaminoglycans on vascular endothelial cells in remote organs, where it can exert noxious effects (31, 38, 49). In fact, a recent study by Vega et al. (46) suggested that XO was released systemically upon hindlimb reperfusion. By inhibiting XO activity with two oral doses of allopurinol, 12 h before and immediately upon hindlimb ischemia, these investigators were able to demonstrate that neutrophil accumulation in the liver was inhibited and plasma alanine transaminase (ALT) never increased above control levels. They contended that XO was the causative factor in the activation of Kupffer cells and hepatic neutrophil accumulation but failed to consider that the timing of their intervention may have diminished their initial insult, leading to possible misinterpretations. As such, we felt it necessary to test the role of XO further in our model of HIR.

Nox enzymes represent a major source of oxidants in the microcirculation. These are membrane-bound enzymes that catalyze the reduction of molecular O2 to O2- and, ultimately H2O2, using NAD(P)H as the electron donor (17, 33). The structure and function of Nox enzymes are well characterized. They are composed of the cytosplasmic subunits p40phox, p47phox, and p67phox and the GTP-binding protein Rac as well as the membrane-bound cytochrome-b558 complex (consisting of p22phox and gp91phox). The cascade of events required for activation of Nox enzymes is thought to be initiated by the phosphorylation of p47phox, enabling it to bind with gp91phox.
(18). This activation results in the well-characterized generation of O$_2^\cdot$-. Most of the components of Nox appear to reside in both phagocytes and the endothelium, including p47$^{\text{phox}}$, p40$^{\text{phox}}$, p67$^{\text{phox}}$ and the cytochrome-b$_558$ complex (4, 29).

Although the classic inhibitor of Nox enzyme activation is diphenylene iodonium, its use is contraindicated in our model because it has been shown to inhibit all flavin-containing enzymes (i.e., cytochrome P-450s, XO, and nitric oxide synthase), which would confound our interpretations (32, 48). Knockout mice lacking p47$^{\text{phox}}$ or gp91$^{\text{phox}}$ also exist, but this would likely affect the initial insult in our model and further lead to misinterpretations. Apocynin (4-hydroxy-3-methoxy-acetophenone), commonly used as an effective inhibitor of O$_2^\cdot$- generation by phagocytic and vascular Nox enzymes, appears to require conversion by H$_2$O$_2$ and myeloperoxidase (MPO) (or some analogous form of peroxidase) for active inhibition (44, 47). However, whether this mechanism of action occurs within the endothelium remains controversial. The chimeric peptide gp91ds-tat is currently the most specific inhibitor of Nox enzymes available. It consists of a 9-amino acid fragment of gp91$^{\text{phox}}$ known to interfere with the interaction between p47$^{\text{phox}}$ and gp91$^{\text{phox}}$. This fragment is linked to a tat site derived from the tat sequence of the human immunodeficiency virus, which allows easy uptake into cells (24, 39). We chose gp91ds-tat to study the contribution of Nox enzymes on remote liver injury, with secondary studies using apocynin.

Although it is generally accepted that an oxidative stress occurs in the liver after HIR, its origin and specific contribution to the parenchymal injury and microvascular dysfunction that ensue has yet to be fully delineated. In this study, we provide evidence suggesting that a Nox-dependent oxidant contributes to the progression of remote liver injury by causing parenchymal damage as well as sinusoidal endothelial cell (SEC) dysfunction. Immunostaining for chlorotyrosine (CT) protein adducts, a footprint for the generation of hypochlorite (HOCI) by neutrophil-derived MPO (10, 23), confirmed that neutrophil-derived oxidants also contributed to the injury. Although Nox inhibition afforded protection to parenchymal and endothelial integrity, it failed to restore microvascular perfusion deficits. Together, these data confirm that oxidative stress is playing a role in the pathogenesis of remote liver injury; it is Nox- and neutrophil-dependent and distinct from the pathogenesis of hepatic microvascular dysfunction.

MATERIALS AND METHODS

Animal protocol. Male C57BL/6J mice (Jackson Laboratory; Bar Harbor, ME) were treated according to the animal care requirements of the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee, and experiments were undertaken according to the manufacturer’s instructions (Thermo Electron; Louisville, CO). Briefly, mice were inoculated with the test reagent for 30 s at 37°C to remove any residual pyruvate in the sample. The test mixture was then read at 340 nm (UV-1601, Shimadzu; Columbia, MD) for 3 min, and the decrease in the rate of absorbance was used to calculate the ALT activity of the sample. The final value was corrected for the dilution of plasma in heparin and expressed as units per liter.

Circulating XO activity. The activity of XO was determined in serum using an Amplex RED Xanthine/XO fluorescence assay kit (Molecular Probes/Invitrogen; Carlsbad, CA) as per the manufacturer’s instructions. Briefly, XO in the sample catalyzes the oxidation of hypoxanthine/xanthine to uric acid and O$_2^\cdot$-. In the reaction mixture, O$_2^\cdot$- spontaneously degrades to H$_2$O$_2$, which, in the presence of horseradish peroxidase, reacts with the Amplex RED reagent to generate the red fluorescent oxidation product resorufin (excitation 530–560 nm and emission 585 nm). The minimal detectable activity is 0.1 mU/ml.

Hyaluronic acid assay. Plasma hyaluronic acid (HA) was measured with an enzyme-linked protein-binding assay using a standard kit (Corgenix; Westminster, CO), and the assay was carried out according to the manufacturer’s instructions. Briefly, wells coated with HA-binding protein were incubated with plasma samples diluted in reaction buffer for 1 h. Diluted plasma samples were then discarded, and the wells were washed four times in PBS (0.01 M). Next, HA-binding protein conjugated to horseradish peroxidase was added to bind to adherent HA in the well. After diluted plasma samples were discarded and the wells were washed, chromogenic substrate was added to develop a colored reaction that was stopped after 30 min by the addition of sulfuric acid (0.36 N). The color intensity was immediately measured with a spectrophotometer (Spectramax 190, Molecular Devices; Sunnyvale, CA) at 450 nm. The concentration of the unknown samples was compared against a standard curve and expressed as nanograms per milliliter. High and low controls were analyzed for quality control purposes, and all samples were run in duplicate.

Chloracetate esterase cytochemistry. Neutrophil staining was carried out according to the manufacturer’s instructions (Sigma). Paraffin-embedded slides were deparaffinized and rehydrated in duplicate steps of xylene, 100% ethanol, 95% ethanol, and double distilled (dd)H$_2$O. Tissue sections were placed into a Coplin jar with the reagent mixture and incubated in a water bath at 37°C for 15 min protected from light. Tissue sections were counterstained with Gill’s hematoxylin and ammonia blue. Glass coverslips were mounted using aqueous mounting media (Faramount, DakoCytomation; Carpinteria, CA). Neutrophils were counted blinded to treatment and expressed as numbers of neutrophils per 10 high-power fields.

Immunohistochemistry. Rehydration and deparaffinization of sections were performed as described above. The generation of the CT antiserum (generously provided by Dr. J. A. Hinson) has been described previously (19). Briefly, tissue sections were incubated at room temperature in Immunopure Peroxidase Suppressor (Pierce Biotechnology; Rockford, IL) for 1 h, rinsed with ddH$_2$O, and then incubated at room temperature in a serum-free protein block (DakoCytomation) to prevent nonspecific binding. After being rinsed with ddH$_2$O, slides were then either incubated in anti-CT serum (1:300) for...
Fig. 1. Progression of remote liver injury is ameliorated by inhibition of NAD(P)H oxidase (Nox) enzymes, not xanthine oxidase (XO). After hindlimb ischemia-reperfusion (HIR), remote liver injury progresses through 6h, as indicated by plasma alanine transaminase (ALT) activity. Treatment with allopurinol after the onset of limb ischemia did not attenuate the injury at any time. In contrast, Nox enzyme inhibition by gp91ds-tat and apocynin protected against the progression of remote liver injury, as evidenced by the significant reduction in ALT at 6h. Data represent means ± SE. *Significantly less than at all other times (P<0.006); †significantly less than 6-h HIR (P<0.002); ‡significantly greater than gp91ds-tat and apocynin at 6-h HIR (P<0.0009).

Fig. 2. Inhibition of Nox enzymes protects against sinusoidal endothelial cell (SEC) dysfunction. Hyaluronic acid (HA) is metabolized by SECs, and its increase in the plasma is an indication of SEC dysfunction. No increases in plasma HA occurred until 6h of HIR. Treatment with allopurinol after the onset of limb ischemia did not attenuate the SEC dysfunction at any time. Nox enzyme inhibition with gp91ds-tat and apocynin resulted in a significant reduction in plasma HA levels, suggesting that it protects against SEC dysfunction. Data represent means ± SE. *Significantly greater than at all other times (P<0.02); †significantly less than allopurinol, control, and scrambled-tat at 6h HIR (P<0.0003).

Table 1. Activity of circulating XO in mice subjected to HIR at different times after reperfusion

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>Allopurinol</th>
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<tbody>
<tr>
<td>30 min</td>
<td>14.14±1.44†</td>
<td>5.11±1.81</td>
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<tr>
<td>3 h</td>
<td>15.46±0.83†</td>
<td>8.41±1.75</td>
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<tr>
<td>6 h</td>
<td>17.93±1.77†</td>
<td>8.41±1.54</td>
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Data are means ± SE (in mU/ml) of circulating xanthine oxidase (XO) activity. HIR, hindlimb ischemia-reperfusion. *Significantly less than at all other times (P<0.009); †significantly greater than the allopurinol group (P<0.003).

RESULTS

Progression of remote hepatic parenchymal injury and SEC dysfunction is ameliorated by inhibiting Nox, not XO. Previously shown to induce acute remote liver injury in a biphasic manner (5, 6, 53), HIR results in a significant increase in plasma ALT by 3h that progressively increases through 6h (Fig. 1). Animals treated with allopurinol after the onset of hindlimb ischemia failed to exhibit any parenchymal protection even though it caused significant inhibition of circulating XO activity (Table 1). In contrast, the inhibition of Nox with gp91ds-tat or apocynin provided significant protection against the progression, but not the early initiation of remote hepatic parenchymal injury. These data suggest that O2·− generation by Nox enzymes, either directly or indirectly, contributes to the hepatic parenchymal injury during HIR.

Because ~90% of circulating HA is eliminated by specific receptor-mediated uptake and catabolism in hepatic SECs (13, 36), increases in its plasma concentration is used as a marker for hepatic SEC dysfunction (2). This is the first study to show that there was significant hepatic SEC dysfunction during the pathogenesis of remote liver injury (Fig. 2). By 6h, there was not satisfied. Sample sizes were 4–6 animals/group. All data were considered significant at P<0.05 with a power of >85%.
nearly a threefold increase in plasma HA in the HIR and allopurinol-treated HIR animals. Inhibition of Nox during HIR completely prevented this rise. Nevertheless, these changes in plasma HA may merely reflect improved sinusoidal perfusion because a more efficient delivery of plasma to SECs could explain the more efficient elimination of HA by SECs. Therefore, differences in sinusoidal perfusion were evaluated for these interventions to rule this explanation out.

Inhibition of Nox and XO fail to improve sinusoidal perfusion after HIR. As previously demonstrated (5, 52), HIR results in significant sinusoidal perfusion deficits indicated by reduced volumetric flow (Fig. 3A). Moreover, there was a significant reduction in sinusoidal diameters and the density of perfused sinusoids after HIR (Fig. 3, B and C). Surprisingly, none of the allopurinol, gp91ds-tat, or apocynin treatments had an effect on these parameters during HIR.

There is a significant increase in hepatic sinusoidal neutrophil accumulation during HIR. Neutrophils significantly accumulated in hepatic sinusoids by 3 h and were present through 6 h of reperfusion after HIR (Table 2). Treatment with allopurinol, gp91ds-tat, and apocynin did not significantly reduce this neutrophil accumulation. In this regard, the interventions provided an environment in which the role of neutrophil-derived oxidants (their degree of activation) could be further delineated without the confounding reduction in the population of sequestered neutrophils that has limited interpretations in previous investigations.

Accumulation of CT protein adducts through 6 h of HIR was reduced by the inhibition of Nox enzymes. The presence of CT protein adducts is a footprint for neutrophil-derived oxidative stress formed through the generation of HOCl from H2O2 and Cl− by MPO (50). With a novel antibody to CT, we were able to demonstrate the presence of neutrophil-derived oxidants during the hepatic oxidative stress and injury that follows HIR (Fig. 4). Intense staining of CT was observed at 6 h in the sinusoids of HIR mice, indicating the generation of HOCl. Preadsorption of the CT antiserum with CT completely prevented all positive staining (data not shown). Moreover, the areas of the liver that showed CT staining were associated with areas of MPO immunoreactivity (Fig. 4). After inhibition of Nox, HIR mice showed complete prevention of hepatic CT staining despite the continued accumulation of neutrophils and sustained immunoreactivity of MPO. There was no reduction in hepatic CT staining at either time point in allopurinol-treated HIR mice (data not shown).

DISCUSSION

Systemic inflammation often results in single- and/or multiple-organ failure (9), and it can occur as a consequence of insults ranging from endotoxemia to blunt-force trauma. In the liver, there is a progression of parenchymal injury through 6 h (5) that occurs concomitantly with microvascular dysfunction (52). Moreover, there is an inflammatory component that

![Fig. 3. Inhibition of either XO or Nox enzymes fail to improve hepatic microvascular dysfunction after HIR. With intravital microscopy, we established that sinusoidal volumetric flow (A) was significantly decreased by 3 h and failed to improve by 6 h in all treatment groups. Moreover, average sinusoidal diameter (B) and functional sinusoidal density (C) were significantly decreased by 3 h with no improvements by 6 h. Neither allopurinol, gp91ds-tat, nor apocynin provided any microvascular protection after HIR. Data represent means ± SE. *Significantly greater than at all other times (P < 0.03).](http://ajpgi.physiology.org/)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Allopurinol</th>
<th>gp91ds-tat</th>
<th>scrambled-tat</th>
<th>Apocynin</th>
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<tr>
<td>Sham (no ischemia)</td>
<td>3.5±1.6*</td>
<td>4.25±1.1*</td>
<td>2.4±0.9*</td>
<td>1.5±0.6*</td>
<td>8.0±3.1*</td>
</tr>
<tr>
<td>HIR 3 h</td>
<td>53.29±7.1</td>
<td>36.2±5.5†</td>
<td>56.5±9.5</td>
<td>58.0±8.8</td>
<td>72.5±12.4</td>
</tr>
<tr>
<td>HIR 6 h</td>
<td>53.4±8.2</td>
<td>37.6±4.1†</td>
<td>58.0±9.1</td>
<td>50.0±7.9</td>
<td>54.33±6.0</td>
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Table 2. Total sinusoidal neutrophil accumulation in livers of mice subjected to HIR at different times after reperfusion using chloracetate esterase

Data are means ± SE of numbers of sinusoidal neutrophils per 10 high-powered fields. gp91ds-tat, inhibitor of NAD(P)H oxidase; scrambled-tat, randomly scrambled 9-amino acid gp91phox docking sequence linked with the tat peptide. *Significantly less than at all other times (P < 0.007); †significantly less than all other treatment groups (P < 0.02).
includes Kupffer cell activation and both sinusoidal and venular neutrophil accumulation (7, 51, 52). Although it is generally accepted that an oxidative stress occurs in the liver after HIR (46), the origins of this stress remain to be fully elucidated. The aim of this study was to determine the source of the oxidant stress and to delineate its contribution to the pathogenesis of remote liver injury in a model of HIR.

In this study, our treatment regimen for allopurinol (immediately upon hindlimb ischemia) did not significantly restore hepatic parenchymal or endothelial integrity, nor did it improve sinusoidal perfusion. We believe this contradiction with the results of Vega et al. (46) can be attributed to one key factor. In their study, Vega et al. treated animals with allopurinol before HIR, which likely altered the initial insult to the hindlimb (i.e., reduced myocyte necrosis upon reperfusion, reduced complement activation, and reduced cytokine release from the hindlimb). In fact, numerous investigations (41, 43) have demonstrated that functional XO is essential for the direct injury to skeletal muscle after HIR. Consequently, treatment with allopurinol before hindlimb ischemia will likely diminish the initial injury beyond XO inhibition and, in turn, alter remote liver damage. With the timing of allopurinol treatment in this investigation, we provide evidence suggesting that XO likely does not contribute to the pathogenesis of remote liver injury despite its significant increase in the plasma during HIR.

To investigate the origin of the hepatic oxidative stress further, we hypothesized that Nox enzymes were involved. Because it is well recognized that Kupffer cells are activated in our model (6), and that there is an acute sequestration of neutrophils (51), we thought it likely that phagocytic Nox O$_2^·$ generation might be a component of the hepatic oxidative stress. Moreover, the stimulation of Nox O$_2^·$ generation in the

![Chlorotyrosine and Myeloperoxidase images]

Fig. 4. Accumulation of chlorotyrosine (CT) protein adducts was prevented by inhibition of Nox enzymes. Immunohistochemistry for CT was not evident in any of the sham mice. No accumulation of CT was evident until 6 h of HIR, with a predominance of sinusoidal staining in the pericentral region. In addition to CT, immunohistochemistry for myeloperoxidase (MPO) on sequential sections showed an association with the CT staining. With Nox enzyme inhibition, CT staining was completely prevented, despite the accumulation of MPO, suggesting that the lack of CT staining in these livers was not a result of diminished MPO. CV, central vein.
liver might also occur via the vascular endothelium (22, 35). The chimeric peptide gp91ds-tat is currently the most effective inhibitor specific to Nox enzymes. It acts by competitively antagonizing the interaction of gp91phox and p47phox (39), which not only suppresses phagocytic Nox O$_2^\cdot$ generation (i.e., Kupffer cells and neutrophils) but also the generation of O$_2^\cdot$ by the vascular endothelium.

Given the lack of cellular specificity for Nox enzyme inhibition, one cannot directly surmise from the study which population of cells is responsible for the oxidant generation. Notwithstanding, because Nox inhibition protects against compromised SEC function, it would suggest that a vascular-derived oxidant stress may be involved in the pathogenesis of remote liver injury. To verify this, we evaluated the presence of CT using immunohistochemistry. CT has been described as the most stable footprint of HOCl production by neutrophil-derived MPO (50), and its presence has been used as a biomarker for MPO-mediated oxidant stress (15, 20). In this study, we demonstrated that CT staining was evident at 6 h in both sinusoids as well as hepatocytes (in a pericentral pattern) and that the staining was associated with MPO. CT staining only returned to basal sham levels with the inhibition of Nox. Because the accumulation of MPO was not altered by Nox inhibition, the reduction in CT accumulation was not due to a reduction in the HOCl-producing enzyme but rather a result of inhibition, the reduction in CT accumulation was not due to a second hit through modulation of signal transduction pathways (26).

In addition to protecting against the progression of hepatic parenchymal injury, Nox enzyme inhibition also prevented the increase in plasma HA, a glycosaminoglycan used as a hallmark for SEC dysfunction. Because HA is specifically metabolized by SECs (13, 36), its increase in the plasma is a strong indication of SEC dysfunction. Moreover, previous studies (12, 14) have shown that alterations in the uptake of HA from the plasma by SECs can occur during various types of liver disease. The present study is the first to show signs of acute hepatic SEC dysfunction in a model of systemic inflammation. This can be caused by either changes in or reductions of the HA receptor on the SEC as well as an absolute compromise to SEC viability (45). We do not expect that the increase in plasma HA is a result of liberation from the reperfused hindlimbs because increases in plasma HA do not occur until 6 h of HIR. In addition, Armstrong and Bell (3) suggested that skeletal muscle ischemia-reperfusion does not cause a significant release of HA.

Besides the functional and structural changes that affect HA uptake by SECs, previous work has suggested that increases in plasma HA may be a result of acute decreases in sinusoidal perfusion (16). As such, we expected improvements in hepatic microvascular perfusion with Nox inhibition based on the observed reductions in plasma HA levels. However, the inhibition of Nox failed to protect against the significant decreases in hepatic parenchymal and endothelial cells during a systemic inflammatory response.

Fig. 5. Proposed mechanism of Nox-dependent oxidative stress on the pathogenesis of remote liver injury. The expression of Nox enzymes can occur in three distinct cell types under acute inflammatory conditions: SECs, Kupffer cells, and neutrophils. The efficient generation of O$_2^\cdot$ by these cells, combined with the rapid dismutation of O$_2^\cdot$ by superoxide dismutase (SOD) to H$_2$O$_2$, provides ample substrate for MPO to form hypochlorite (HOCl). The physical nature of SECs (i.e., gaps and fenestrae) provides easy access for MPO, released from neutrophils, to hepatocytes to exert its noxious effects. By inhibiting Nox enzymes, we block the generation of O$_2^\cdot$ by these cells and thereby prevent the downstream effects and ameliorate the damage to hepatic parenchymal and endothelial cells during a systemic inflammatory response.
in hepatic sinusoidal volumetric flow. Moreover, both functional sinusoidal density and sinusoidal diameter remained reduced. These data confirm our previous work showing that the mechanisms participating in the development of hepatic microvascular dysfunction and compromised parenchymal viability during a systemic inflammatory stress are distinct and disparate (5). As such, our results lend credence to the premise that multiple factors act in a temporally and spatially distinct manner during the pathogenesis of remote liver injury.

The fact that Nox inhibition improved SEC function while failing to improve perfusion is intriguing. The facts that microvascular dysfunction occurred as early as 3 h and signs of SEC dysfunction were not present until 6 h suggests that perfusion deficits are not directly dependent on the function of SECs (as it pertains to the uptake and catalysis of HA). Moreover, the fact that the inhibition of Nox enzymes failed to improve perfusion negates the argument that improved delivery of HA to SECs was responsible for the decrease in plasma HA levels. Nonetheless, this dysfunction indicates that SECs are undergoing some degree of stress. The increase in sinusoidal tone, which has been shown previously (52), suggests that there is a vasomotor component involved in the perfusion deficits. This increase in tone, along with the presence of less deformable red blood cells and activated neutrophils, may also participate in the microvascular dysfunction that occurs.

The preferential accumulation of neutrophils in sinusoids of smaller diameter has been shown previously (52). However, there were only slight alterations in the numbers of sequestered hepatic neutrophils with each of the given interventions. As such, we contend that the degree of sequestered neutrophils does not necessarily correlate with the degree of injury. In fact, it has been suggested by others that neutrophil sequestration could be reduced by as much as 60% before a significant protective effect against neutrophil-mediated injury could be detected (27). This suggests that it is not necessarily the number of sequestered neutrophils that determines their cytotoxicity, but it is their state of activation that matters (8, 25). Notably, the maintained neutrophil sequestration that occurred with Nox inhibition did not cause increases in parenchymal injury or interfere with the ability of SECs to metabolize HA. This suggests that it is the generation of oxidants, and not the accumulation of inflammatory cells, that is involved in the compromised hepatic parenchymal and endothelial integrity that occurs after HIR.

In conclusion, it should be recognized that inflammatory cells are not only accumulating in the liver after HIR but are also generating an oxidant stress via Nox-dependent O$_2^-$ generation and MPO. The vascular endothelium is also likely involved in the manifestation of this Nox-dependent oxidant stress, which appears to be linked to both the progression of hepatic parenchymal injury and SEC dysfunction. However, the suppression of HOCl formation that occurs with Nox inhibition does not protect against the initial injury at 3 h, nor the microvascular dysfunction. A more thorough understanding of what occurs early in remote liver injury will provide a better understanding into the different mechanisms participating in the transition from organ injury to organ failure.

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REFERENCES