Microcirculatory perfusion deficits are not essential for remote parenchymal injury within the liver

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Brock, Robert W., Michael W. Carson, Kenneth A. Harris, and Richard F. Potter. Microcirculatory perfusion deficits are not essential for remote parenchymal injury within the liver. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G55–G60, 1999.—A normotensive model of hindlimb ischemia-reperfusion in Wistar rats was used to test the hypothesis that microvascular perfusion deficits contribute to the initiation of remote hepatic injury during a systemic inflammatory response. Animals were randomly assigned to one of three groups: 4 h of ischemia with 6 h of reperfusion (I/R-6; n = 4), 4 h of ischemia with 3 h of reperfusion (I/R-3; n = 5), or no ischemia (naive; n = 5). With intravital fluorescence microscopy, propidium iodide (PI; 0.05 mg/100 g body wt) was injected for the in vivo labeling of lethally injured hepatocytes (number/102 mm3). PI-positive hepatocytes increased progressively over the 6-h period (naive 32.9 ± 7.8 vs. I/R-3 92.8 ± 11.5 vs. I/R-6 232 ± 39.2), with no difference between perportal and pericentral regions of the lobule. Additionally, a significant decrease in continuously perfused sinusoids (naive 70.0 ± 1.5 vs. I/R-3 65.0 ± 1.0 vs. I/R-6 48.8 ± 0.9%) was measured. Regional sinusoidal perfusion differences were only observed after 3 h of limb reperfusion. Indirect measures of hepatocellular injury using alanine transaminase levels support the progressive nature of hepatic parenchymal injury (0 h 57.8 ± 6.5 vs. 3 h 115.3 ± 20.7 vs. 6 h 125.6 ± 19.5 U/l). Evidence from this study suggests that remote hepatic parenchymal injury occurs early and progresses after the induction of a systemic inflammatory response and that microvascular perfusion deficits are not essential for the initiation of such injury.

intravital microscopy; ischemia-reperfusion; remote injury; microcirculation

Both clinical (16) and experimental (21, 28) evidence suggest that either infection or trauma without infection may initiate a systemic inflammatory response. It is also generally accepted that injury to organs remote from a focus of initial injury is the leading cause of death within intensive care units across North America (7, 9). However, the mechanisms leading to the initiation of these remote organ injuries remain elusive, despite intensive study.

On the basis of various experimental models of sepsis, evidence suggests that microvascular perfusion deficits, including increased flow heterogeneity, are one of the first changes to occur within remote organs (6, 8, 10, 18, 22). Thus it is conceivable that significant reductions in microvascular perfusion within these organs may result in ongoing hypoxic stress and thus predispose the parenchyma to injury. Few studies have attempted to link such microvascular perfusion deficits to remote parenchymal injury.

Studies employing direct injury to the liver, via ischemia-reperfusion or shock, have suggested that microcirculatory failure was a prerequisite for parenchymal injury (3, 4, 17, 27). However, conflicting reports exist with regard to the contribution of flow deficits and parenchymal injury. For example, Machiedo et al. (12) showed that microvascular flow deficits preceded hepatocyte injury after endotoxin administration, whereas Wang et al. (25) demonstrated unaltered hepatic microcirculatory flow despite hepatocellular damage. This conflict may be a consequence of either differences in the model used (i.e., shock) or the indirect nature of the methods used for measurement of perfusion and injury.

The purpose of the present study was to determine if microvascular perfusion deficits occurred within the liver during the early stages of a systemic inflammatory response in the absence of shock and if these perfusion deficits contributed to the initiation of hepatocellular injury. To accomplish our goal, we employed the use of a normotensive model of systemic inflammation initiated by hindlimb ischemia-reperfusion. By using measures of whole organ injury and intravital fluorescence microscopy, we provide direct evidence that microcirculatory perfusion deficits and hepatocellular injury are initiated concurrently during a systemic inflammatory response, but the occurrence of these changes were not correlated.

MATERIALS AND METHODS

Animals. All animals (male Wistar rats, 200–250 g) used in this study were randomly assigned to either the 6-h naive group (no ischemia; n = 5), the 3-h reperfusion group (I/R-3; n = 5), or the 6-h reperfusion group (I/R-6; n = 4). It was believed that the 6-h naive group was a representative control due to the temporal nature of the parameters observed. The experimental protocols described herein were undertaken in accordance with the criteria outlined by the Canadian Council on Animal Care and approved by the University of Western Ontario Council on Animal Care.

Surgical procedure. Animals were anesthetized by inhalational isoflurane (5% induction, 2% surgery, and 1% maintenance; Abbott Laboratories). During anesthesia, the left carotid artery was cannulated for continuous monitoring of systemic arterial pressure, for blood sampling, and to allow for the application of fluorescent vital dyes. The right jugular vein was also cannulated for administration of the analgesic fentanyl citrate (2 µg·100 g body wt-1·h-1). All animals were chronically instrumented with a Harvard swivel and fluid

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resuscitated with normal saline (2–4 ml/h) throughout the study. Bilateral hindlimb ischemia was induced for 4 h with the application of a tourniquet (#4 silk; Johnson & Johnson Medical) above the greater trochanter of each leg. Tourniquets were removed just before recovery from anesthesia. Naïve animals underwent the same protocol, with the ischemic event omitted.

Intravital videomicroscopy. After either the 3- or 6-h period of limb reperfusion, animals were reanesthetized with isoflu- rane, and the left lobe of the liver was exposed and reflected into a warmed saline bath (37°C) on the stage of an inverted microscope (Diaphot-TMD; Nikon). Once placed in position, the liver was covered with plastic film (Saran; Dow Chemical) to prevent dehydration and minimize the influences of respiratory movement and atmospheric O2. Random views (10–13) of the microcirculation, near the surface of the liver, were observed using a ×20 objective lens (CF Achromat 20/0.4; Nikon) with illumination provided by fiber-optic light guides (Intralux 5000; Volpi). In all cases, the randomly selected fields of view were limited to areas 5–10 mm from the edge of the lobe. Images were projected onto a charge-coupled device video camera (Dage-MTI) and recorded on videotape for offline analysis using an SVHS video recorder (VC).

Offline video analysis of lethal hepatocyte injury and sinusoidal perfusion. Severely injured hepatocytes were labeled in vivo with an intra-arterial bolus of the fluorescent vital dye propidium iodide (PI; 0.05 mg/100 g body wt) just before the 3- and 6-h limb reperfusion times. Nuclei labeled with PI were used to identify hepatocytes that were irreversibly damaged (10). The PI fluorescence was viewed by exposure to excitation wavelengths of 510–560 nm with an emission barrier filter >590 nm. An index of lethal hepatocyte injury was determined offline and expressed as the number of PI-labeled nuclei per unit volume of tissue (PI-labeled nuclei/10⁻³ mm³).

Sinusoidal perfusion was determined using a stereological point-counting grid that provided a >95% confidence that the density of points counted was proportional to the density of sinusoids within the area of interest. A sinusoid was counted if a point landed within its dimensions. A continuously perfused sinusoid (CPS) was defined as one that possessed continuous red blood cell (RBC) flow for the 1-min observation period. If a perfused sinusoid demonstrated RBC flow that stopped at least once during the 1-min observation period, it was deemed an intermittently perfused sinusoid (IPS). Non-perfused sinusoids (NPS) were represented by any sinusoid that did not have RBC flow throughout the entire observation period. Each perfusion parameter was expressed as a proportion of the total number of sinusoids counted.

Lethal hepatocyte injury and sinusoidal perfusion were measured in both the periportal and pericentral regions of the hepatic lobule. To determine if a relationship existed between hepatocyte injury and perfusion deficits, correlational analyses were performed. Thus the number of PI-labeled hepatocytes was plotted as a function of CPS within individual microscopic fields for the total liver lobule and both functional regions (periportal and pericentral regions) at 3 and 6 h of limb reperfusion.

Liver enzyme assay and PO2. In a separate set of animals, measurements of the liver-specific enzyme alanine transaminase (ALT) were made from samples drawn from the carotid artery at three different time points: 1) 0 h of limb reperfusion (n = 10), 2) 3 h of limb reperfusion (n = 10), and 3) 6 h of limb reperfusion (n = 10). ALT activity (U/l) was tested using a standard spectrophotometric technique. Furthermore, blood samples were collected from the carotid artery for measurement of PO2. Arterial PO2 was determined using a blood gas analyzer (Radiometer).

Fig. 1. After 4 h of bilateral hindlimb ischemia, mean arterial pressures (A) were maintained in ischemia-reperfused (I/R) animals (●) compared with those of naive animals (○). Arterial PO2 levels (B) between experimental groups were not significantly different when measured at 0 h of reperfusion (open bars) and at 6 h of reperfusion (solid bars). Differences between times exist because of application of supplemental O2 (~40%) during anesthesia. Data represent means ± SE. *P < 0.05 vs. 0 h reperfusion.
hepatocellular damage. Four hours of remote ischemia resulted in a significant elevation in ALT, exhibiting a progressive increase over the first 3 h of limb reperfusion (P < 0.05; Fig. 2). ALT levels were not different between the third and sixth hours of reperfusion. Measures of ALT during the 4-h ischemic period did not show any significant changes (data not shown).

Cellular index of lethal hepatocyte injury. By labeling damaged hepatocytes with the fluorescent vital dye PI, we measured a progressive increase in lethal hepatocyte injury over the 6-h limb reperfusion period for the entire liver lobule (periportal plus pericentral). These measures showed significant and progressive increases in lethal hepatocyte injury in both the I/R-3 and I/R-6 groups compared with the naive group (P < 0.05; Fig. 3A). The total number of hepatocytes within the volume of interest was estimated with the use of fluorescence microscopy and the permeable dye bisbenzamide. These estimates suggest that <5% of hepatocytes were lethally injured. When our analyses segregated the liver lobule into its functionally distinct zones, the periportal and pericentral regions, a significant progression in the degree of PI-labeled hepatocytes was evident in both regions (P < 0.05; Fig. 3B). Thus no regional differences in injury were measured.

Index of sinusoidal perfusion. Throughout the entire liver lobule, CPS declined progressively from naive to I/R-3 and finally to I/R-6 (P < 0.05; Fig. 4A). Although such a progressive decline in CPS was evident in the periportal region (P < 0.05; Fig. 4B) at 3 h, it was not present in the pericentral region, where a significant decline in CPS occurred only after 6 h of reperfusion (P < 0.05). Another regional difference in perfusion was the significantly lower CPS and significantly greater IPS in the periportal region compared with the pericentral region for all groups (P < 0.05; Fig. 4B and Table 1). Increases in IPS and NPS for both the I/R-3 and I/R-6 groups account for the decline in CPS in both regions of the liver lobule (P < 0.05; Table 1).

Role of sinusoidal perfusion in lethal hepatocyte injury. No significant relationship was found between lethal hepatocyte injury and sinusoidal perfusion for either the entire liver lobule or the functional regions within the lobule (Figs. 5 and 6).

**DISCUSSION**

We believe this study to be novel in that it provides direct evidence of remote hepatic injury during the early pathogenesis of a systemic inflammatory response. Such injury was characterized by significant hepatocellular injury with concurrent, albeit regionally distinct, reductions in microcirculatory perfusion. Although not tested in the present study, hepatocellular dysfunction may be more severe than would be expected by the small percentage of lethally injured cells. Postschismic elevations in ALT suggest the presence of a large population of injured hepatocytes whose function may be compromised. Thus one might predict that a measure of hepatocellular function may show a much greater decrease than the 5% predicted by our direct measure of cell death.
In contrast to the increased levels of hepatocellular injury, progressive perfusion deficits were only found to be present within the periportal region. Taken together, our results of hepatocellular injury and death, coincident with increased microvascular perfusion heterogeneity, suggest that the liver is vulnerable during the early stages of a systemic inflammatory response. Furthermore, such results absolve the absolute reduction in hepatic microcirculatory perfusion as a mecha-

![Fig. 4.](image)

**Fig. 4.** Throughout entire liver lobule (A), percentage of continuously perfused sinusoids (CPS) progressively declined from naive to I/R-3 and I/R-6 animals. Although this decline was present in periportal region (naive [open bars], I/R-3 [hatched bars], and I/R-6 [solid bars]) after zonal segregation of lobule (B), pericentral region did not exhibit significant deficits until 6th h of reperfusion. Data represents means ± SE. *P < 0.05 vs. naive group; †P < 0.05 vs. 0 h of reperfusion; ‡P < 0.05 vs. periportal region.

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<th>IPS, %</th>
<th>NPS, %</th>
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<tr>
<td>Periportal</td>
<td>Pericentral</td>
</tr>
<tr>
<td>Naive</td>
<td>32.61 ± 1.46</td>
</tr>
<tr>
<td>I/R-3</td>
<td>36.42 ± 1.53</td>
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<tr>
<td>I/R-6</td>
<td>45.69 ± 1.23†</td>
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Values are means ± SE. I/R-3 and I/R-6, 3 and 6 h, respectively, of ischemia-reperfusion; IPS, intermittently perfused sinusoids; NPS, nonperfused sinusoids. *P < 0.05 vs. naive group; †P < 0.05 vs. I/R-3 group; ‡P < 0.05 vs. periportal region.

![Fig. 5.](image)

**Fig. 5.** Correlation between degree of lethal hepatocyte injury and percentage of CPS after hindlimb I/R in naive (○), I/R-3 (●), and I/R-6 (▲) for entire (combined) liver lobule. No significant relationship appeared to exist. R, Pearson product moment correlation coefficient.

![Fig. 6.](image)

**Fig. 6.** Relationships between degree of lethal hepatocyte injury and percentage of CPS within individual microscopic fields after hindlimb I/R in naive (○), I/R-3 (●), and I/R-6 (▲) for different functional zones of liver lobule: periportal region (A) and pericentral region (B). Again, no significant relationship was exhibited between microvascular perfusion and parenchymal injury.
nism leading to the initiation of lethal hepatocellular injury during a systemic inflammatory process. Interpretation of the results in many studies of remote hepatic injury are complicated by the possibility that the injury may be induced in a direct fashion, in addition to remote processes. For example, models such as cecal ligation and perforation (30) or intestinal I/R (19, 23), may directly compromise liver integrity via bacterial peritonitis, physical manipulation, and/or organ hypoperfusion. Although hindlimb I/R has been used to study remote injury in the gut (5, 29), lung (21, 28), and liver (26, 27), these studies failed to account for the possibility of ongoing hypoxic stress induced by circulatory shock. The use of a fluid-resuscitated model of hindlimb I/R, such as the one used in the present study, provides several advantages. It reduces the possibility of direct manipulation of the liver or direct contamination of the peritoneum and prevents liver hypoperfusion, controlling for changes to either systemic arterial pressures or arterial PO2. Thus we believe our resuscitated model provided a more reliable means to test specific mechanisms thought to be important in the initiation of remote hepatic injury.

Recently, MacPhee and colleagues (14) measured intermittent velocity profiles in normal rat liver sinusoids and proposed that flow alterations become more heterogeneous during pathological states. This hypothesis was later supported by Vollmar et al. (24), who showed greater perfusion heterogeneity in rat liver subjected to I/R compared with control animals. Our results substantiate this hypothesis, particularly during the early pathogenesis of remote hepatic injury, without the complications of systemic shock or an ongoing hypoxic stress.

Although mechanisms leading to reduced microvascular perfusion within the liver were not tested here, one can speculate that the perfusion heterogeneity observed may result from sinusoidal narrowing caused by edema (2) or vasoconstriction induced by liver-specific pericytes (i.e., Ito cells). Ito cells have been shown to increase their contractile activity in response to endothelin-1 or reductions in nitric oxide (1, 11, 17). The regional flow heterogeneity within the liver lobule may be related to the distinct functional and structural differences between the perportal and pericentral regions. Sinusoids within the perportal region have been shown to be smaller, more tortuous, and branch more frequently than those of the pericentral region (15, 20). The nature of these regional differences may further predispose the perportal region to plugging by various cells, especially stiffened RBCs and/or activated leukocytes. The resident macrophage population of the liver (i.e., Kupffer cells) may also contribute to regional perfusion differences, because they are located primarily in the perportal region and their activation is thought to contribute to alterations in sinusoidal blood flow (13).

As a whole, lobular microvascular dysfunction appeared to occur concurrently with parenchymal injury, supporting a possible functional perfusion deficit-injury relationship similar to that proposed for direct hepatic reperfusion injury (3, 17). However, when the lobule was segregated into zones, the pericentral region did not show evidence of perfusion deficits despite significant lethal hepatocellular injury. Our temporal and spatial measures of perfusion and lethal injury failed to support a functional relationship between such measures. Had the lobule not been segregated into its distinct zones, the occurrence of hepatocellular injury in the absence of microvascular perfusion deficits would have remained undetected.

Linear regression analyses have been employed to describe the relationship between hepatic microvascular perfusion deficits and parenchymal injury (3, 17). This analysis may not be appropriate if the nature of the perfusion deficit was progressive, as shown in our study within the periportal zone. If the progressive decline in perfusion lead to hypoxic stress, then a correlation between perfusion and injury might be best described using exponential decay analysis. However, application of such analysis in the present study again failed to show any significant relationship between perfusion and parenchymal injury. The liver possesses a dense three-dimensional vascular system; consequently, perfusion deficits would need to be severe or spatially localized for such a relationship to be observed. The absence of perfusion deficits despite hepato cellular injury in the pericentral zone and the seemingly random occurrence of sinusoids with either no flow or intermittent RBC flow within the entire lobule provide further support that perfusion deficits and injury are unrelated. Such conclusions are strengthened by the fact that in no case were PI-positive cells (lethally injured hepatocytes) associated with immediately adjacent sinusoids devoid of perfusion.

We believe the disparity between the results of the present study (i.e., the lack of a significant relationship between microvascular perfusion and lethal hepatocellular injury) and those reported previously (3, 17) likely reflect subtle differences between the mechanisms leading to direct versus remote injury. For example, liver injury occurs within minutes of reperfusion after direct liver ischemia. Such injury is commonly believed to reflect the cellular changes imposed by the hypoxic stress created by the ischemic period and thus predispose the tissue to reactive oxygen metabolite-induced injury on reperfusion. However, in our model there was no evidence of direct hypoxic stress, and thus the mechanisms leading to liver injury during a systemic inflammatory response likely reflect a cascade of events initiated by the reperfusion of the remote tissue. The specific events and mediators of such remote liver injury remain an area of intensive study.

We believe this study is original in that it provides direct evidence of hepatic parenchymal injury and microvascular perfusion deficits early and progressively after a remote inflammatory stimulus in the absence of circulatory shock. It is unlikely, however, that hepatic microvascular perfusion deficits initiate the lethal hepatocyte injury that occurs during the early stages of this systemic inflammatory process. Whether different mechanisms are responsible for caus-
ing remote microvascular perfusion deficits and parenchymal injury remains to be tested.

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