Molecular mechanisms of leukocyte recruitment in postischemic liver microcirculation

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Infiltrating neutrophils have been implicated (18, 22, 28, 35, 43) as key mediators of ischemia-reperfusion injury associated with numerous organs, including the intestine, heart, brain, skeletal muscle, and liver. Many investigators (14, 30) have taken advantage of the translucent properties of the mesenteric microvasculature to directly visualize neutrophil-blood vessel wall interactions. This approach has provided a paradigm for neutrophil recruitment in ischemia-reperfusion. Immediately after ischemia (1–10 min of reperfusion), neutrophils begin to roll slowly along postcapillary venules mainly as a result of the rapid expression of presynthesized P-selectin on the endothelial surface (20, 30, 31). Several mediators have been evoked as potential inducers of neutrophil rolling during ischemia-reperfusion, including leukotrienes and oxidants in rodent systems (1, 8) and thrombin in larger mammals (42). Indeed, in humans, thrombin has been shown (12, 27, 54) to be a potent activator of endothelium to induce neutrophil rolling. The rolling cells reduce their rolling velocity significantly and begin to adhere by 10 min of reperfusion, and this persists for at least the next 2 h (30). This adhesion can be reversed by either inhibiting the β2-integrin (CD18) or giving animals antithrombin III, an endogenous inhibitor of thrombin (15, 30, 31).

Similar selectin-dependent rolling and integrin-dependent adhesion mechanisms of neutrophil-endothelial cell interactions have also been reported for skin (39, 40), muscle (23, 26), and heart (17, 24); however, these tenets do not always apply to hold true for organs such as the liver. The liver, with its portal circulation, very extensive sinusoidal network, and associated slow flow rates, supports adhesion in postsinusoidal venules as well as in sinusoids (50). In fact, the sinusoids appear to be the dominant site of leukocyte sequestration in certain inflammatory conditions (21, 50). The importance of this site-specific recruitment is that the aforementioned paradigm of selectin initiating rolling does not appear to be relevant to the liver sinusoids (21, 50). Bacterial products [including lipopolysaccharide (LPS) and N-formylmethionyl-leucyl-phenylalanine], cytokines [including tumor necrosis factor (TNF)], and proinflammatory mediators [including leukotriene C4 or platelet-activating factor (PAF)] induced profound leukocyte recruitment in the sinusoids independent of selectins (6, 11, 50). In fact, to date, inhibition of selectins has not provided any beneficial effect in liver inflammation (9, 21, 50) with one consistent exception: ischemia-reperfusion (37, 45, 52, 53).

Indeed, in dramatic contrast, many groups have observed that inhibition of P-selectin (37, 45, 53), the ligand for P-selectin (PSGL-1) (2), and L-selectin (36,
all inhibit leukocyte recruitment into postischemic liver as assessed by biochemical or histological approaches. Furthermore, inhibition of multiple selectins with sialyl Lewisα (a ligand for selectins) or a small molecule selectin inhibitor resulted in reduced neutrophil recruitment in the postischemic liver (38, 44). One might infer from these results that, unlike other inflammatory conditions of the liver, ischemia-reperfusion the majority of leukocytes are recruited in postsinusoidal and not sinusoidal vessels or alternatively that anti-selectin therapy prevents leukocyte recruitment by some unexpected mechanism in sinusoids. We therefore examined leukocyte-endothelium interactions in the liver microcirculation in vivo using intravital microscopy and asked two simple questions: does ischemia-reperfusion of the liver induce leukocyte recruitment in the sinusoids and post-sinusoidal venules, and does general inhibition of selectins reduce cell recruitment in either or both microvascular compartments? We used two approaches: 1) direct inhibition of selectins with an anti-selectin fucoidan and 2) inhibition of thrombin (hirudin), the mediator responsible for selectin upregulation in intestinal ischemia-reperfusion (42). Because of the unexpected observation that anti-selectin and antithrombin therapy prevented adhesion in both sinusoids and post-sinusoidal vessels, we examined whether the anti-selectin therapy could also prevent leukocyte recruitment associated with a localized inflammatory condition (superfusion of only the liver microvasculature with endotoxin) in the feline liver. Finally, occlusion of blood flow to the liver during ischemia could affect the intestine, which could cause liver inflammation. In a final series of experiments, we examined whether anti-selectin therapy blocked leukocyte recruitment in hepatic ischemia-reperfusion when the intestine was completely removed from the portal vasculature.

**METHODS**

*Surgery for intravital microscopy.* The experimental preparation for liver in this study is similar to that used previously for cat mesenteric intravital microscopy (30). Animal protocols were approved by the University of Calgary Animal Care Committee and met the Canadian Guidelines for Animal Research. Briefly, cats (1.2–2.4 kg) were fasted for 24 h and initially anesthetized with ketamine hydrochloride (75 mg im). The jugular vein was cannulated, and anesthesia was maintained by the administration of pentobarbital sodium at 37°C using an infrared heat lamp. All exposed tissues were warmed bicarbonate-buffered saline (pH 7.4) bubbled with 5% CO₂ and 95% N₂. The small intestine remained in place inside the abdominal wall, and the exposed liver was covered with Saran wrap (Dow Corning, Midland, MI) to restrict lateral and vertical motion at the plane of focus. The temperature of the pedestal was maintained at 37°C with a constant temperature circulator (model 80, Fisher Scientific, Pittsburgh, PA). Instead of conventional illumination, oblique lighting provided by a fiber-optic source was positioned at ∼45° to the optical axis. The image of the microcirculatory bed was recorded using a video camera (Dage MTI N C-07, Michigan City, IN) and a video recorder (NV98950; Panasonic).

Postsinusoidal venules (25- to 40-μm diameter; 250-μm length) with associated sinusoids were selected for each study. The venular diameter was measured on or offline using a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, TX). The number of rolling and adherent leukocytes was determined offline during playback analysis. Rolling leukocytes were defined as white blood cells that moved at a velocity less than that of erythrocytes in a given vessel. The number of rolling leukocytes (flux) was counted using frame-by-frame analysis. To obtain a complete leukocyte rolling velocity profile, the rolling velocity of all leukocytes entering the vessel was measured. A leukocyte was defined as adherent to venular endothelium if it remained stationary for >30 s. Adherent cells were measured at 10-min intervals as described in Experimental protocol and expressed as the number per 100-μm length of venule.

**Experimental protocol.** In the first series of experiments, all aforementioned parameters were allowed to stabilize for 30 min and baseline measurements of blood pressure and vessel diameter were obtained. Leukocyte parameters, including leukocyte rolling velocity, flux of rolling leukocytes, and leukocyte adhesion, were measured. The superior mesenteric artery was occluded for 1 h, and the above-mentioned parameters were measured again. The clamp was released, and hemodynamic and leukocyte parameters were measured at 10, 60, and 120 min.

In a second series of experiments, the selectin-binding polysaccharide fucoidan, a homopolymer of sulfated L-fucose, was administered to animals (25 mg/kg) 5 min before reperfusion. We (30) have previously reported that this dose of fucoidan induced an optimal reduction in leukocyte rolling in the mesentery without causing neutropenia. Fucoidan administered at 1 mg/kg caused only 60% inhibition of leukocyte rolling (30). For comparison to the ischemia-reperfusion model, leukocyte recruitment was also examined in a local model of hepatic inflammation, i.e., continuous superfusion of the liver with LPS (1 μg/ml) over 4 h. This concentration of LPS causes no notable systemic effects, including no change in blood pressure, but induces a profound increase in leukocyte accumulation in the feline hepatic microvasculature.

Previous work from our laboratory (42) has revealed that thrombin is the main inducer of P-selectin-dependent leukocyte rolling and adhesion in the feline mesentery. However, in the previous study (42), the results were deemed somewhat inconclusive, because antithrombin III was used and this molecule has been shown to have effects in addition to antithrombin effects (19). To determine whether thrombin was also an important inducer of leukocyte recruitment in
feline liver microvasculature, we administered recombinant hirudin (0.4 mg iv bolus over 20 s and then 0.15 mg kg⁻¹ h⁻¹ continuous iv infusion) as previously described (25). This concentration has been shown to inhibit leukocyte-endothelial interactions induced by extracorporeal circulation.

A major unresolved issue in previous studies, including those mentioned above, is that occlusion of blood flow to the liver inevitably reduces blood flow to the intestine. Therefore, in a final series of experiments, the superior mesenteric blood flow was rerouted from the superior mesenteric artery to the superior mesenteric vein via an extracorporeal circuit and the small and large intestine was removed. The portal pressure was set at 9 mmHg, which resulted in a blood flow of ~20 ml/min or lower. This is the portal pressure consistently measured in cats (32) and in our own findings (unpublished). This caused complete removal of blood flow to or from the small bowel so that occlusion of the superior mesenteric blood flow followed by reperfusion impacted only on the liver. In a second group of animals, the same surgical intervention was performed and fucoidan was again administered 5 min before reperfusion.

Statistics. The data were analyzed using standard statistical analysis, i.e., ANOVA and Student’s t-test with Bonferroni’s correction for multiple comparisons when appropriate. Values are means ± SE. Statistical significance was set at \( P < 0.05 \).

RESULTS

Figure 1, top, demonstrates that the number of rolling leukocytes in postsinusoidal venules of the liver ranged from 25 to 40 cells/min. Occlusion of the superior mesenteric artery maintained a small amount of blood flow through the liver microvasculature (presumably via the hepatic artery). This allowed for continued rolling of leukocytes through postsinusoidal venules. At the time of reperfusion, the number of rolling leukocytes increased approximately fourfold to ~110–140 cells/min at 60 min of reperfusion, remaining at this level for the next hour (Fig. 1, top). The rolling velocity of these cells was between 30 and 35 \( \mu \text{m/s} \) and decreased by ~30% during ischemia and reperfusion (Fig. 1, middle). The number of adherent leukocytes is illustrated in Fig. 1, bottom. Under basal conditions, fewer than three cells adhered per 100-\( \mu \text{m} \)-long venule in the postsinusoidal vessels. During ischemia and reperfusion, there was a very significant increase in the number of adherent leukocytes, and this persisted for the full 2 h.

Figure 1 also illustrates the responses to fucoidan, a general selectin inhibitor. Fucoidan reduced the number of rolling cells by >95% (Fig. 1, top). The few cells that rolled did so with the same rolling velocity observed in the group of animals that did not receive fucoidan (Fig. 1, middle). Finally, fucoidan significantly reduced the number of adherent leukocytes (Fig. 1, bottom).

Figure 2 highlights the large increase in leukocyte sequestration or adhesion within sinusoids. Within the field of view, one sees ~6–10 sinusoidal vessels; under control conditions, very few of these vessels have any adhering leukocytes. During ischemia and reperfusion, a very significant number of these vessels have adherent leukocytes. Between 10 and 15 leukocytes can be seen adhering in sinusoids. Although anti-selectin therapy has not reduced leukocyte recruitment into sinusoids with LPS, TNF, or PAF in rodent models (6, 11, 50), during ischemia-reperfusion, fucoidan induced a very profound reduction in the number of sinusoidal leukocytes (Fig. 2). In direct contrast, when LPS was delivered locally (superfusion) to affect only the liver and not the intestine, LPS-induced leukocyte recruitment into sinusoids was not affected by fucoidan (Fig. 3). Leukocytes accumulated over time in sinusoids in response to LPS, and pretreating animals with fu-
Fucoidan induced at least as many leukocytes in the liver sinusoids over the 4-h experimental protocol (Fig. 3).

It is noteworthy that the number of rolling cells induced by LPS in postsinusoidal venules was again significantly reduced (~80%) with fucoidan (Fig. 4, top), albeit not to the same degree as in ischemia-reperfusion. During LPS superfusion, a very significant decrease in leukocyte rolling velocity was also noted, and this parameter was not affected by fucoidan (Fig. 4, middle). The number of adherent leukocytes in postsinusoidal vessels was increased with the time of LPS superfusion, and in this particular case, the number of adherent cells was not affected by fucoidan (Fig. 4, bottom).

In a previous study, we (42) reported that thrombin contributed very significantly to ischemia-reperfusion-induced leukocyte rolling and adhesion in the mesenteric microvasculature. Therefore, we next tested the specific thrombin inhibitor hirudin. Figure 5, top, dem-
onstrates that the increased leukocyte rolling observed during ischemia-reperfusion was not affected by hirudin as the number of rolling leukocytes in postsinusoidal venules increased approximately fourfold in both experimental groups. Figure 5, middle, reveals that the 30% decrease in rolling velocity was not observed with hirudin treatment. Leukocyte adhesion in postsinusoidal vessels was very significantly reduced by hirudin during reperfusion (Fig. 5, bottom). In fact, the number of adherent cells dropped from ~15 cells/100-μm-long venule to <5 cells/100-μm long venule.

Figure 6 demonstrates a very similar pattern in sinusoids. Whereas ischemia and reperfusion induced at least 10 adherent cells in these microvessels, a 70% reduction in this parameter was noted after hirudin administration.

The fucoidan results could imply that leukocyte recruitment was reduced in sinusoids as a result of direct inhibition of selectin function in the sinusoids or due to inhibition of injury upstream in the intestine. Indeed, ischemia-reperfusion of the intestine induces leukocyte adhesion in liver sinusoids and intestinal microvessels, and the latter is dependent on selectins (19, 20). Therefore, in the final series of experiments, we developed a new model, wherein the intestine was extirpated before ischemia-reperfusion. This allowed us to induce ischemia-reperfusion exclusively in the liver. Interestingly, in this model, removal of the intestine resulted in a very significant decrease in the basal number of rolling leukocytes (Fig. 1 vs. Fig. 7). This may reflect the continuous release of some activating agents in the intestine that enhance leukocyte rolling in postsinusoidal venules, even under basal conditions. In this model, we were able to induce a significant increase in leukocyte rolling and adhesion in postsinusoidal venules and induced many leukocytes to sequester in sinusoids (Figs. 7 and 8). However, when fucoidan was added in this system, the results were very different from the ischemia-reperfusion data generated with an intact intestine and far more in line with the endotoxin data. Although fucoidan inhibited many of the rolling cells in the postsinusoidal vessels (Fig. 7, top), significant numbers of leukocytes still adhered (Fig. 7, bottom). Most importantly, fucoidan did not reduce the number of leukocytes within sinusoids (Fig. 8).
DISCUSSION

In this study, we demonstrated that leukocyte recruitment after ischemia-reperfusion and local exposure of endotoxin to liver result in the firm adhesion of leukocytes in both the sinusoids and postsinusoidal venules. Whereas the leukocyte adhesion induced by ischemia-reperfusion in both the sinusoids and postsi-

![Graph](image1)

**Fig. 7.** In this series of experiments, the intestine was removed before ischemia. The flux of rolling leukocytes (top), their rolling velocity (middle), and the number of adherent leukocytes (bottom) before (Con) and after 60 min of ischemia and 2 h of reperfusion are shown. One group received fucoidan (n = 4), and the other group received vehicle (n = 4). *P < 0.05 relative to respective control value; +P < 0.05 relative to corresponding untreated value.

nusoidal venules was inhibitable by a general anti-selectin inhibitor, the same intervention had absolutely no effect on leukocyte adhesion in the liver during local superfusion of endotoxin. In addition, we demonstrated that inhibition of thrombin with hirudin after ischemia-reperfusion also prevented leukocyte adhesion in both the sinusoids and postsinusoidal venules. However, unlike previous studies (42) in the mesentery, which revealed that an inhibitor of thrombin (antithrombin III) prevented rolling and hence subsequent adhesion in postischemic venules, in the current study in the liver the antithrombin drug hirudin was unable to prevent leukocyte rolling despite having a very profound impact on leukocyte adhesion. This clearly demonstrates some interesting organ-specific differences in the mediators that induce leukocyte rolling in the liver vs. the mesentery.

Anti-selectin therapy prevented adhesion in the ischemic and reperfused postsinusoidal venules. The diameters of these vessels are sufficiently large that leukocytes are not in intimate contact with the vessel wall and hence require active tethering to the vessel wall via selectins. Therefore, the initial selectin-dependent tethering and rolling in these postsinusoidal venules are prerequisites for subsequent adhesion. Inhibition of selectins has therefore presumably led to reduced adhesion in these vessels. More surprising, however, is our finding that anti-selectin therapy also reduced leukocyte adhesion in sinusoids. In these vessels, there is no rolling per se as the diameter of the sinusoids is not significantly different from the diameter of the leukocytes (50). Therefore, the leukocytes are already in close contact with the vessel walls and anti-selectin therapy should not have blocked adhesion. Indeed, there are reports (9) of a lack of selectin
expression in sinusoids and studies (9), including our own (6, 11, 50), showing that inhibition of selectins does not prevent leukocyte accumulation into sinusoids.

However, numerous studies (37, 45, 52, 53) have reported that anti-selectin therapy results in either reduced leukocyte infiltration into postischemic liver or reduced injury after hepatic ischemia-reperfusion. One possible explanation may be that ischemia-reperfusion induces the majority of leukocytes to be recruited into postsinusoidal and not sinusoidal vessels. However, our intravital microscopy study clearly demonstrated a robust leukocyte response in the sinusoids as well as in postsinusoidal vessels. An alternative explanation may be that anti-selectin therapy inhibited leukocyte recruitment in postsinusoidal venules, which accounts for the partial inhibition in total leukocyte recruitment after ischemia-reperfusion. However, general anti-selectin therapy inhibited adhesion within both the sinusoids and postsinusoidal vessels. Clearly, ischemia-reperfusion induces leukocyte recruitment in both the sinusoids and postsinusoidal vessels, and anti-selectin therapy blocks both events.

One very valid criticism of all models of ischemia-reperfusion of the liver is that the small intestine is in series with the liver and interruption of liver blood flow inevitably disrupts intestinal blood flow. Therefore, when the superior mesenteric artery is occluded, both the intestine and liver become ischemic, and when both organs are reperfused, it is possible that various toxic agents released into the intestinal bloodstream could affect the liver. Because much of the intestinal injury has been shown (13, 14, 18, 29) to be dependent on accumulating neutrophils, it is conceivable that the delivery of anti-selectins prevented intestinal injury, which prevented the release of toxic products into the bloodstream and thereby prevented neutrophil accumulation into the liver. Indeed, ischemia-reperfusion of the intestine induces profound leukostasis in the liver (19), an event not noted in P-selectin-deficient mice. To our knowledge, the current study directly tests this hypothesis for the first time with the complete removal of the intestine so that it did not affect the liver microcirculation. The results revealed that ischemia-reperfusion of the liver, in the absence of the intestine, induced leukocyte rolling and adhesion in postsinusoidal vessels and adhesion in sinusoids, and although the rolling was still sensitive to fucoidan, the adhesion in both types of vessels could not be inhibited by anti-selectin therapy. These data support the view that selectins per se do not mediate adhesion in the sinusoids and perhaps that our results, and those of others (2, 36, 37, 45, 53), in which a decrease in leukocyte recruitment with anti-selectin therapy was observed may reflect indirect protection in the intestine.

The fact that we observed very effective inhibition of leukocyte adhesion in sinusoids with fucoidan in our ischemia-reperfusion model also suggests that in this model the intestine accounts for the majority of the leukocyte recruitment in liver. If this is the case, then we should not have observed any leukocyte recruitment when ischemia-reperfusion was induced in the liver and the intestine was removed. One possible explanation for the increased recruitment of leukocytes in the model in which the intestine was removed is that with the intestine intact the majority of the blood entering the liver postsischemia was venous. We know that low oxygen-containing blood causes no posts ischemic tissue inflammation (16). In our model in which the intestine was removed, we reperfused with oxygenated (arterial) blood, which caused ample leukocyte recruitment. This permitted us to assess whether direct ischemia-reperfusion in the liver led to selectin-dependent leukocyte recruitment in sinusoids. The answer was clear: selectins were not required for adhesion of leukocytes in posts ischemic sinusoids. It is also worth noting that our data were entirely consistent with our other local liver inflammation model (endotoxin model), wherein selectins also played no role.

Although the aim of this study was not to identify the mechanisms by which leukocytes are recruited into sinusoids of posts ischemic intestine, several mechanisms may exist. First, during ischemia-reperfusion of the intestine, epithelial barrier disruption occurs, leading to translocation of enteric flora and LPS into the portal circulation (7). In addition, certain proinflammatory mediators, including PAF (33), activated complement fragments (51), and cytokines (4), have been shown to be released from the posts ischemic intestine into the systemic circulation and lymph. In fact, lymph from reperfused intestine has both leukocyte- and endothelial cell-activating and cytotoxic properties (49). In addition, plasma xanthine oxidase (a generator of free radicals) is greatly increased after intestinal ischemia-reperfusion (47), and this enzyme has the capacity to bind endothelium in distal vascular beds to induce injury (46). Finally, inappropriate activation of leukocytes as they pass through the posts ischemic intestine may also contribute to selectin-dependent leukocyte-platelet aggregates, which may contribute to leukocyte recruitment in the hepatic microvasculature (5, 31).

Thrombin, the terminal protease of the coagulation pathway, is best known for its ability to cleave fibrinogen and activate platelets. However, there is increasing evidence that thrombin also can directly induce the rapid expression of presynthesized P-selectin from Weibel-Palade bodies of endothelium to induce leukocyte rolling (10, 12, 54). In addition, thrombin can induce the rapid synthesis of the phospholipid, PAF, to induce firm adhesion (34). Indeed, exposure of endothelium to thrombin within minutes induces P-selectin-dependent leukocyte rolling and CD18 integrin-dependent leukocyte adhesion (27, 41). We reported (42) that the increased rolling and adhesion observed in mesenteric vessels after ischemia-reperfusion was dependent on thrombin, because antithrombin III inhibited both rolling and adhesion. In contrast, the current data demonstrate that inhibition of thrombin with the specific antithrombin inhibitor hirudin prevented leukocyte adhesion but not rolling in the liver postsinusoidal venules. This suggests that thrombin is involved in inducing integrin-dependent firm adhesion...
but not selectin-dependent rolling in liver ischemia-reperfusion. If the intestine is indeed responsible for the increased leukocyte rolling and adhesion in the postischemic intestinal venules, then thrombin-independent mediators released from the intestine must have induced leukocyte rolling in the liver. Clearly, the difference in the liver (the current study) and the mesentery (42) suggests that each organ may have its own series of mediators responsible for inducing leukocyte rolling. However, the beneficial effect of antithrombin III in the mesentery is subject to criticism since antithrombin III has also been shown to release pro-tacyclin (48). Pro-tacyclin has been shown (3) to have potent anti-adhesive properties, raising the possibility that thrombin does not contribute to ischemia-reperfusion-induced leukocyte recruitment. The present study clearly demonstrates that a more specific inhibitor of thrombin, hirudin, does indeed prevent leukocyte accumulation in the postischemic liver, highlighting the importance of thrombin as an important proadhesive molecule in ischemia-reperfusion.

In summary, our data suggest that the beneficial effects of anti-selectin therapy observed in liver ischemia-reperfusion but not in liver inflammation associated with local endotoxin and other conditions are related to the ability of the anti-selectin therapy to prevent accumulation of the leukocytes within the sinusoids. This reduction in leukocyte accumulation may be due to reduced inflammation in the intestine rather than in the liver per se, because in a model in which we removed the intestine, ischemia-reperfusion-induced leukocyte recruitment in sinusoids was not dependent on selectins.

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