Roles for C-X-C chemokines and C5a in lung injury after hindlimb ischemia-reperfusion

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Roles for C-X-C chemokines and C5a in lung injury after hindlimb ischemia-reperfusion. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L57–L63, 1999.—We evaluated the roles of the C-X-C chemokines cytokine-induced neutrophil chemotactant (CINC) and macrophage inflammatory protein-2 (MIP-2) as well as the complement activation product C5a in development of lung injury after hindlimb ischemia-reperfusion in rats. During reperfusion, CD11b and CD18, but not CD11a, were upregulated on neutrophils [bronchoalveolar lavage (BAL) and blood] and lung macrophages. BAL levels of CINC and MIP-2 were increased during the ischemic and reperfusion periods. Treatment with either anti-CINC or anti-MIP-2 IgG significantly reduced lung vascular permeability and decreased lung myeloperoxidase content by 93 and 68%, respectively (P < 0.05). During the same period, there were significant increases in serum C5a-related neutrophil chemotactic activity. Treatment with anti-C5a decreased lung vascular permeability, lung myeloperoxidase, and BAL CINC by 51, 58, and 23%, respectively (P < 0.05). The data suggest that the C-X-C chemokines CINC and MIP-2 as well as the complement activation product C5a are required for lung neutrophil recruitment and full induction of lung injury after hindlimb ischemia-reperfusion in rats.

The initial phase (<1 h of reperfusion) is independent of neutrophils, with tissue injury being mediated primarily by resident macrophage populations (3, 9). The later phase of local organ injury (>1 h of reperfusion) is dependent on tissue recruitment of neutrophils because depletion of neutrophils before ischemia greatly reduces reperfusion injury in lung and liver (12, 23). Activation of the complement system contributes to neutrophil accumulation and organ injury after ischemia-reperfusion in numerous organs (6, 10, 15, 31, 32). With the use of a model of lung inflammation induced by IgG immune complexes, it has been shown that the complement activation product C5a modulates lung neutrophil recruitment by enhancing pulmonary expression of tumor necrosis factor (TNF)-α and intercellular adhesion molecule (ICAM)-1 (16). A specific role for C5a in organ neutrophil recruitment and injury induced by ischemia-reperfusion has not been demonstrated.

The mechanisms of neutrophil recruitment to lung after remote organ ischemia-reperfusion are largely unknown. After hepatic ischemia-reperfusion, serum levels of TNF-α increase (2). It has been suggested that liver-derived TNF-α induces the pulmonary production of the C-X-C chemokine epithelium-derived neutrophil attractant-78, which mediates lung neutrophil recruitment (1). Two potent C-X-C chemokines, cytokine-induced neutrophil chemoattractant (CINC) and macrophage inflammatory protein-2 (MIP-2), have been shown to be required for pulmonary neutrophil recruitment and lung injury after intrapulmonary deposition of IgG immune complexes (25). Acute lung injury induced by airway instillation of bacterial lipopolysaccharide has also been shown to be MIP-2 dependent (21). However, chemokine involvement in lung injury induced by hindlimb ischemia-reperfusion has not been determined.

In the current studies, we sought to determine whether CINC, MIP-2, and C5a contributed to pulmonary neutrophil recruitment and lung injury induced by hindlimb ischemia-reperfusion. The data demonstrate lung production of CINC and MIP-2 and systemic generation of C5a during both the ischemic and reperfusion periods. Additionally, treatment with blocking antibodies to CINC, MIP-2, and C5a cause reductions in lung neutrophil recruitment and lung injury. These findings suggest that CINC, MIP-2, and C5a are necessary for the full induction of lung injury induced by hindlimb ischemia-reperfusion. The role of C5a may be, in part, that it facilitates generation of C-X-C chemokines.
MATERIALS AND METHODS

Ischemia-reperfusion model. Pathogen-free male Long-Evans rats (275–300 g; Charles River Breeding Laboratories, Portage, MI) were used for all studies. Intrapitoneal injec-
tions of ketamine (100–150 mg/kg) together with xylazine (3 mg/kg) were given for sedation and anesthesia. The animals were anesthetized throughout the entire procedure with injections of one-fourth of the initial dose administered every 20–30 min. Tourniquets with pressure sufficient to block arterial blood flow were placed on both hindlimbs proximal to the trochanter major muscle mass in anesthetized animals. After 4 h of ischemia, the tourniquet was released and reperfusion was allowed to occur for the next 4–8 h. Control animals received the same type of anesthesia together with tourniquets that were not tightened.

Measurement of lung vascular permeability. Before release of the tourniquet, a trace amount (0.5 µCi) of 125I-labeled bovine serum albumin was injected intravenously. After 4 h of reperfusion, the rats were exsanguinated, the pulmonary circulation was flushed with 10 ml of phosphate-buffered saline (PBS) and Hanks’ by pulmonary artery injection, and the lungs were surgically dissected. The extent of lung injury was quantitated by calculating the lung permeability index (the ratio of radioactivity in the lung to radioactivity present in 1 ml of blood obtained at the time of death). It is possible that this measurement could be biased by retention of some blood in the lungs and that not all blood had been removed by perfusion of lungs via the pulmonary artery. In an earlier report (24) employing the same model, pulmonary vascular damage was verified by the presence of intra-alveolar hemorrhage and edema.

Measurement of neutrophil accumulation. Four hours after reperfusion, whole lungs were surgically dissected and immediately frozen in liquid nitrogen. Lungs were homogenized and sonicated, and myeloperoxidase (MPO) content was measured with a colorimetric assay described elsewhere (29).

In vivo blocking of C5a and C-X-C chemokines. Polyclonal goat IgG anti-rat C5a and anti-rat CINC and polyclonal rabbit IgG anti-rat MIP-2 were produced and purified as previously described (16, 25). The anti-C5a was double-affinity purified to remove cross-reactivity with rat MIP-2. Similarly, anti-rat MIP-2 antibody was purified to remove cross-reactivity with rat CINC (25). For in vivo blockade, 400 µg of IgG were injected intravenously just before release of the tourniquets. Companion positive control animals received 400 µg of purified IgG from preimmune goat or rabbit serum.

CINC and MIP-2 content in bronchoalveolar lavage fluids. At the time of death, 5 ml of PBS were instilled and withdrawn three times from the lungs through an intratra-
echal cannula. The bronchoalveolar lavage (BAL) fluids were centrifuged at 400 g for 15 min. Supernatant fluids were collected, and an anti-protease cocktail ([in mg/ml] 1 leupeptin, 1 aprotinin, 10 soybean trypsin inhibitor, and 1 pepstatin) was added. Measurements of CINC and MIP-2 in BAL fluids were performed with enzyme-linked immunosorbent assays as previously described (25).

Neutrophil chemotaxis assay. Normal human blood was collected in vials containing citrate (anti-coagulant citrate dextrose solution USP Formula A, Baxter, Deerfield, IL). Neutrophils were isolated by Ficoll and dextran sedimentation (Pharmacia Biotech, Uppsala, Sweden). Remaining red blood cells were acid lysed. The neutrophils were washed, suspended in PBS (without Ca2+ and Mg2+) containing 0.1% BSA (endotoxin free), and labeled with 2’,7’bis-(2-carboxyethy1)-5-(and-6)-(carboxyfluorescein) acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR). The cells were washed again and resuspended in Hanks’ balanced salt solution with 0.1% BSA (–1 ng/mg endotoxin) at a final concentration of 5 × 106 cells/ml. Bottom compartments of the chemotaxis chamber (Neuro Probe, Cabin John, MD) were loaded with 30 µl of rat serum (diluted 1:10 in Hanks’ balanced salt solution) obtained at the indicated times during hindlimb ischemia-reperfusion. Formyl-Met-Leu-Phe at concentrations of 10–2 to 10–10 M was used as a standard reference chemoattractant. Formyl-Met-Leu-Phe concentrations of the cell suspension were applied to each top well, which contained a polycarbonate membrane with a pore size of 3 µm. The chambers were incubated for 30 min at 37°C. The filters were removed, and nonmigrating cells were wiped off. Fluorescence on the bottom side of each filter was read on a CytoFluor II multiwell plate reader (PerSeptive Biosystems, Framing-
ham, MA) at 630 nm. Data in Fig. 3 are expressed as a percentage of formyl-Met-Leu-Phe chemotactic activity.

RESULTS

CINC and MIP-2 in BAL fluids. Levels of CINC and MIP-2 in BAL fluids were measured throughout the periods of ischemia and reperfusion. Rather surprisingly, at all of the time points (1, 2, and 4 h) after the onset of ischemia, there were significant increases in BAL levels of both CINC (Fig. 1A) and MIP-2 (Fig. 1B) compared with time 0 of ischemia. BAL levels of CINC and MIP-2 were significantly increased at all time points of reperfusion (1, 2, 4, and 8 h). Thus, although the lung itself did not undergo ischemia, pulmonary chemokine expression was increased during the ischemic period and remained elevated during reperfusion of the hindlimbs.
Protective effects of anti-CINC and anti-MIP-2 on lung injury. In vivo blockade of CINC or MIP-2 was achieved by intravenous injection of 400 µg of anti-CINC or anti-MIP-2 immediately before hindlimb reperfusion. Positive control animals were infused with 400 µg of preimmune IgG. Treatment with anti-CINC caused a 71% reduction in vascular permeability ($P < 0.007; \text{Fig. 2A}$) and a 93% reduction in MPO content ($P < 0.046; \text{Fig. 2B}$). Similarly, treatment with anti-MIP-2 caused a 71% reduction in vascular permeability ($P < 0.004; \text{Fig. 2A}$) and a 68% reduction in MPO content ($P < 0.047; \text{Fig. 2B}$). Thus, there appear to be requirements for both CINC and MIP-2 for lung neutrophil recruitment and subsequent development of lung injury after hindlimb ischemia and reperfusion.

Serum chemotactic activity for neutrophils during ischemia and reperfusion. In these studies, we assessed to what extent C5a was appearing in the plasma of rats undergoing ischemia and reperfusion of hindlimbs. Neutrophil chemotactic activity of serum retrieved from a central indwelling venous catheter was assessed at the time points indicated during ischemia and reperfusion (Fig. 3). Serum samples were collected at 1, 2, and 4 h during ischemia (4 h) and at 1, 2, 4, and 8 h during the 8-h period of reperfusion. Samples were analyzed for neutrophil chemotactic activity in the presence of preimmune goat IgG or goat anti-rat C5a IgG (each at 5 µg/ml). In samples containing preimmune IgG, there was significant chemotactic activity after 1 and 4 h of ischemia and after 1, 4, and 8 h of reperfusion. Virtually all chemotactic activity could be suppressed in the presence of anti-rat C5a (Fig. 3). Thus systemic activation of complement begins during hindlimb ischemia and continues during reperfusion.

Attenuation of ischemia-reperfusion-induced lung injury by anti-C5a. As shown in Fig. 4, the intravenous injection of 400 µg of anti-rat C5a just before release of the tourniquets significantly reduced the extent of lung injury measured 4 h after the initiation of hindlimb reperfusion. Pulmonary vascular permeability (as determined by extravascular leak of 125I-labeled albumin) was decreased by 51% ($P = 0.023; \text{Fig. 4A}$), and lung content of MPO was reduced by 59% ($P = 0.007; \text{Fig. 4B}$). The levels of CINC in BAL fluids were reduced by 23% ($P = 0.040; \text{Fig. 4C}$). Treatment of rats with anti-C5a failed to cause a significant reduction in BAL levels of MIP-2 (data not shown). Therefore, it seems that C5a is required for the full development of lung injury.
injury after hindlimb ischemia-reperfusion, at least in part, by augmenting the production of CINC and increasing lung neutrophil recruitment. However, it is apparent that C5a has actions beyond its effects on intrapulmonary generation of CINC.

Adhesion molecule expression on neutrophils and macrophages. Flow cytometric analysis of CD11a, CD11b, and CD18 on blood neutrophils and BAL neutrophils and macrophages are shown in Fig. 5. In all cases, negative control cells (cells obtained from nonmanipulated rats) showed relatively low levels of $\beta_2$-integrins. Surface expression of CD11b and CD18 on BAL macrophages increased significantly by 1 h of reperfusion, diminished at 2 h, and returned to baseline by 4 h of reperfusion (Fig. 5A). Hindlimb ischemia-reperfusion did not alter macrophage expression of CD11a from control values. Surface expression of CD11a, CD11b, and CD18 on BAL neutrophils was unchanged at the initiation of reperfusion (time 0) compared with negative control cells (Fig. 5B). After 1 h of reperfusion, however, expression of CD11b and CD18 on BAL neutrophils was significantly increased. Enhanced expression of CD11b and CD18 was persistent throughout the 4-h period of reperfusion. No elevation in CD11a of BAL neutrophils occurred at any time point. The patterns of $\beta_2$-integrin expression on blood neutrophils (Fig. 5C) were very similar to those found on BAL neutrophils, with elevations peaking at 1 h of reperfusion and remaining above normal throughout the 4-h period of reperfusion. Thus, during the reperfusion period, Mac-1 (CD11b/CD18), but not lymphocyte function-associated antigen-1 (CD11a/CD18), was elevated on lung macrophages as well as on blood and BAL neutrophils. The extent to which blocking of C5a, CINC, or MIP-2 would reduce upregulated levels of CD11b/CD18 on these phagocytic cells remains to be determined.

DISCUSSION

In liver and lung, the condition of ischemia activates resident macrophages, resulting in increased production of proinflammatory cytokines, chemokines, and adhesion molecules (1, 3, 4). On reperfusion, systemic complement activation occurs, causing activation of circulating neutrophils, which migrate to the previously ischemic organ (6, 10, 15, 31, 32). The recruitment of neutrophils requires the concerted efforts of chemokines and adhesion molecules. The process of neutrophil recruitment includes adhesion to the vascular endothelium in postischemic tissues via CD18- and/or ICAM-1-dependent mechanisms (4, 11). During hepatic ischemia-reperfusion, CINC and MIP-2 contribute significantly to neutrophil accumulation into postischemic liver (7, 14). However, in organs remote to the
site of ischemia and reperfusion, the factors responsible for the recruitment of neutrophils are unclear. After hepatic ischemia-reperfusion, it has been reported that liver-derived TNF-α is released into the systemic circulation and induces the pulmonary production of the C-X-C chemokine epithelial-derived neutrophil attractant-78, which contributes to lung neutrophil recruitment (1). An additional study (32) suggests that complement activation products may mediate neutrophil recruitment into remote organs (lung) after ischemia-reperfusion.

Our data further delineate the mechanisms of neutrophil recruitment to lung after hindlimb ischemia-reperfusion. Quite unexpectedly, we found increased levels of CINC and MIP-2 in BAL fluids even during the ischemic period. It is interesting to note that under these conditions BAL levels of CINC and MIP-2 were similar to levels found after intrapulmonary deposition of IgG immune complexes, powerful stimuli for chemokine production in lung (25). The precise mechanism by which hindlimb ischemia induces pulmonary production of CINC and MIP-2 remains elusive. At least in the case of CINC, generation of C5a during ischemia could stimulate pulmonary production of this chemokine. The data in Fig. 3 indicate that C5a was present in serum even during the period of ischemia. It seems likely that systemic activation of complement was occurring. This could be due to release of some complement-activating product from the ischemic limb. Alternatively, perhaps there was release of neuropeptides or other factors that directly or indirectly activate the complement system to generate C5a. The fact that treatment with antibody to C5a attenuated levels of CINC in BAL fluids suggests that C5a may be partially responsible for increases in CINC during hindlimb ischemia (and reperfusion). The mediators that regulate MIP-2 production in lung during hindlimb ischemia remain to be determined. It is unlikely that upregulation of MIP-2 (or CINC) is caused by circulating proinflammatory cytokines because serum levels of TNF-α, interleukin-1, and interleukin-6 are undetectable during the ischemic period in this model (22).

Despite greatly increased levels of CINC and MIP-2 in BAL fluids during hindlimb ischemia, neutrophils do not accumulate in the lung during this time (23, 24). This may be due to the fact that before hindlimb reperfusion there was no evidence of increased surface expression of β2-integrins on circulating blood neutrophils. Requirements for β2-integrins and their primary endothelial ligand ICAM-1 have been demonstrated in this model for the full induction of lung injury (22). The current data suggest that upregulation of β2-integrins on circulating neutrophils may be a prerequisite for neutrophil recruitment into lung. At least during the ischemic period, it seems that increased pulmonary chemokine production by itself is not sufficient to induce neutrophil accumulation. On the other hand, during reperfusion, when pulmonary chemokine production is increased and β2-integrin expression is greatly increased on blood neutrophils and BAL neutrophils and macrophages, treatment with blocking antibodies to CINC or MIP-2 greatly suppressed the lung inflammatory response to hindlimb ischemia-reperfusion. Blockade of CINC and MIP-2 resulted in 93 and 68% reductions, respectively, in lung MPO content, indicating that CINC and MIP-2 are essential for lung recruitment of neutrophils after hindlimb ischemia-reperfusion.

These studies suggest a specific role for C5a in the development of lung injury after hindlimb ischemia-reperfusion. It appears that, among other things, C5a generated from the systemic activation of complement activates cells in the lung to increase production of CINC. Treatment with antibody to C5a attenuated lung production of CINC and reduced lung MPO content and vascular permeability induced by ischemia-reperfusion. In this model of ischemia-reperfusion, it seems likely that C5a in some manner facilitates the full expression of CINC and MIP-2 in BAL fluids and that these C-X-C chemokines, in turn, cause activation of blood and BAL neutrophils and BAL macrophages. Alternatively, it is possible that CD11b/CD18 expression on these phagocytic cells is directly related to stimulation by C5a. Which of these possibilities may pertain still needs to be determined. In a model of myocardial ischemia-reperfusion, it has been suggested that C5a directly contributes to neutrophil recruitment (8). Furthermore, in lung injury induced by IgG immune complexes, C5a potentiates lung production of TNF-α and upregulates pulmonary vascular ICAM-1 expression (16). Thus it is possible that C5a contributes in a similar fashion to the lung recruitment of neutrophils after hindlimb ischemia-reperfusion.
In summary, we have demonstrated that pulmonary production of the C-X-C chemokines CINC and MIP-2 is increased during hindlimb ischemia and reperfusion in rats. Blockade of either of these chemokines significantly reduces lung neutrophil accumulation and lung injury. Blockade of C5a attenuated the pulmonary production of CINC and reduced the extent of lung injury. These studies identify CINC, MIP-2, and C5a as important mediators of remote organ neutrophil recruitment and injury after ischemia-reperfusion of hindlimbs. The chain of events that links these mediator requirements remains to be defined.

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