In Vivo Correlation of Neutrophil Receptor Expression, Ischemia-Reperfusion Injury, and Selective 5-Lipoxygenase Inhibition in Guinea Pigs

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**Objective:** To determine whether selective 5-lipoxygenase (5-LO) inhibition decreases expression of adhesion molecules (β2 integrins) on systemic neutrophils, decreases neutrophil infiltration in ischemic flap tissue, and improves flap survival.

**Design:** A randomized, controlled study of 91 adult female Hartley guinea pigs divided into 3 survival groups, 4 neutrophil assay groups, 1 sham group, and 1 control group. Ischemia of varying duration and reperfusion was induced in island flank skin flaps. The treated groups received zileuton, a 5-LO inhibitor, orally during flap ischemia. After reperfusion, systemic neutrophil receptor expression, neutrophil infiltration, and flap survival were measured. Surface receptor molecules on neutrophils from whole blood samples obtained via transcardiac puncture were analyzed using monoclonal antibodies and cell-associated fluorescence. Neutrophil infiltration into a distal 1 cm² of flap tissue was assessed using myeloperoxidase antibodies. Flap survival was determined within 7 days of surgery.

**Results:** Untreated flaps with 10 hours of ischemia underwent total necrosis. Treated 2- and 10-hour ischemic flaps survived intact. A significant main effect of the drug treatment was detected using analysis of variance (P<.001). Neutrophil receptor detection in the untreated groups undergoing 2 and 10 hours of ischemia was significantly increased compared with that in the treated groups with the same ischemia times. Skin neutrophil infiltration was significantly decreased in the treated groups.

**Conclusions:** Systemic administration of a 5-LO inhibitor is effective in reducing ischemia-reperfusion injury in flap tissue. Our data indicate that there is a significant reduction in neutrophil receptor expression with administration of 5-LO, reducing the priming of systemic neutrophils from circulating cytokines.


SELECTIVE INHIBITION of 5-lipoxygenase (5-LO) prevents activation of stimulated neutrophils in vitro.1 Activation involves up-regulation of neutrophil receptors on the cell surface, most importantly, the β, subfamily, including LFA-1 (CD11a/CD18) and CR3 (CD11b/CD18).2 These receptors interact with intercellular adhesion molecules on endothelial cells to initiate adhesion and transmigration of activated neutrophils into tissue parenchyma, the rate-limiting step in reperfusion injury.3,4 The effectiveness of therapy with 5-LO inhibitors may be attributed to the putative role that 5-LO serves in the intracellular cascade of events that leads to receptor up-regulation.5 Reducing receptor expression will attenuate reperfusion injury and improve ischemic tissue survival because of the central role of neutrophils in ischemia-reperfusion (I/R) injury.6,5 Anti-inflammatory drug therapy has been successful in reducing I/R injury in several tissue types by decreasing neutrophil infiltration.7 Therapy with phospholipase A2 and lipoxygenase inhibitors is effective in reducing I/R injury, whereas use of cyclooxygenase inhibitors is ineffective.8,9 The molecular basis for the effectiveness of 5-LO inhibition has been partially elucidated in a few in vitro studies of stimulated neutrophils showing reduced surface receptor expression.1 Demonstraton of the effectiveness of 5-LO inhibition in reducing I/R injury while correlating the findings with CD18 expression requires an in vivo model of flap ischemia. This study was done to determine whether selective 5-LO inhibition and decreased expression of adhesion molecules (β2 integrins) on systemic neutrophils correlates with reduced neutrophil infiltration in ischemic flap tissue and improved flap survival. Dorsal island guinea pig flaps underwent varying durations of ischemia, and the animals in the treatment groups were given the selective 5-LO inhibitor zileuton (N-1{1-benzo(h)thien-
MATERIALS AND METHODS

Ninety-one adult female Hartley guinea pigs (600-700 g) were divided into 3 survival groups, 4 neutrophil assay groups, 1 sham group, and 1 control group. Ischemia-reperfusion was induced in island flank skin flaps. All animals in the neutrophil assay groups underwent transcardiac puncture (TCP) and lethal exsanguination to obtain a sufficient volume of blood for the neutrophil assay.

All animals (except those in the control group) underwent a series of basic procedures. They were anesthetized with intramuscular injections of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (8 mg/kg). The flank skin was shaved with an electric razor and prepared with povidone-iodine solution. A dorsal island skin flap (8 × 4 cm) modeled after that of Hirigoyen et al11 was developed based on the superficial circumflex iliac pedicle. To induce flap ischemia, the artery and vein were isolated for manipulation and were individually clamped using Weck microvascular temporary occlusion clips (8 × 15-mm blades). Cessation of flow was confirmed using the operative microscope by the Acland maneuver.12 The flap edge was sutured using interrupted 3-0 nylon (Ethicon, Johnson and Johnson, Somerville, NJ) sutures. The animals were placed on a hot water circulation pad (42°C) to maintain body temperature. Ischemia duration was either 2 or 10 hours (except for the control and sham groups). Two hours before reperfusion, zileuton (10 mg/kg in a hydroxyethylcellulose vehicle and 1 mL/2 mg of zileuton) or the vehicle alone (equivalent volume) was administered orally through a pediatric feeding tube followed by a 1-mL sterile water flush. The schedule and dosing of zileuton treatment was based on the experience of Malo et al13 using dose-response curves measuring dynamic lung compliance in guinea pigs exposed to aerosolized meclofenamic acid and mepyramine maleate, wherein they found that administration of a single oral dose was effective for more than 12 hours. Reperfusion was initiated by releasing the inferior sutures over the pedicle and releasing the clamps. Resumption of flow was again confirmed by the Acland maneuver. The opened portion of the flap was sutured, and 2 hours after reperfusion, a full-thickness skin biopsy specimen (1 × 1 cm) was taken from the craniodorsal corner (a biopsy specimen was taken in the sham group also). Animals in the neutrophil assay groups were exsanguinated by TCP and immediately killed. Animals in the survival groups were allowed to recover on a hot water pad until posture could be maintained and then were transferred to the animal care room. They were caged separately and given food and water ad libitum. Buprenorphine hydrochloride (0.05 mg/kg) was given every 2 hours to control pain in the postoperative period. The proportion of flap survival was determined using a method described previously by Dolan et al.14 Flaps were evaluated daily for up to 7 days. If the flap was totally necrotic before day 7, the animal was killed. This animal protocol was approved by the Boston University School of Medicine’s Institutional Animal Care and Use Committee, Boston, Mass. Analysis of variance and the Bonferroni multiple comparison tests were used to analyze the neutrophil receptor data, and the Kruskal-Wallis test was used to analyze the neutrophil infiltration data.

GROUPS

The animals with 2-hour ischemic flaps (1) were given vehicle and underwent TCP (n = 9; 2 hours, without drug, untreated), (2) were given zileuton and underwent TCP (n = 10; 2 hours, with drug, treated), or (3) were given vehicle and allowed to survive (n = 10; 2 hours, survival group). The animals with 10-hour ischemic flaps (1) were given vehicle and underwent TCP (n = 10; 10 hours, without drug, untreated), (2) were given zileuton and underwent TCP (n = 10; 10 hours, with drug, treated), (3) were given vehicle and were allowed to survive (n = 10; 10 hours, survival group). The schedule and dosing of zileuton treatment was based on the experience of Malo et al13 using dose-response curves measuring dynamic lung compliance in guinea pigs exposed to aerosolized meclofenamic acid and mepyramine maleate, wherein they found that administration of a single oral dose was effective for more than 12 hours. Reperfusion was initiated by releasing the inferior sutures over the pedicle and releasing the clamps. Resumption of flow was again confirmed by the Acland maneuver. The opened portion of the flap was sutured, and 2 hours after reperfusion, a full-thickness skin biopsy specimen (1 × 1 cm) was taken from the craniodorsal corner (a biopsy specimen was taken in the sham group also). Animals in the neutrophil assay groups were exsanguinated by TCP and immediately killed. Animals in the survival groups were allowed to recover on a hot water pad until posture could be maintained and then were transferred to the animal care room. They were caged separately and given food and water ad libitum. Buprenorphine hydrochloride (0.05 mg/kg) was given every 2 hours to control pain in the postoperative period. The proportion of flap survival was determined using a method described previously by Dolan et al.14 Flaps were evaluated daily for up to 7 days. If the flap was totally necrotic before day 7, the animal was killed. This animal protocol was approved by the Boston University School of Medicine’s Institutional Animal Care and Use Committee, Boston, Mass. Analysis of variance and the Bonferroni multiple comparison tests were used to analyze the neutrophil receptor data, and the Kruskal-Wallis test was used to analyze the neutrophil infiltration data.

RESULTS

FLAP SURVIVAL

Flap survival was an all or none phenomenon. Flaps that had undergone 10 hours of ischemia without drug (10 hours, survival group A) all underwent total necrosis by 7 days. Flaps that had undergone 2 hours of ischemia with drug (2 hours, with drug) and 10 hours of ischemia with drug (10 hours, with drug) all survived intact. The 2 hours, with drug group was done to test the possibility of an adverse effect of the drug.

PERIPHERAL BLOOD NEUTROPHIL ANTIBODY ASSAY

The neutrophil fluorescence counts were divided by the control group’s mean fluorescence count; therefore, each measure represented a ratio of this baseline value. Neutrophil yield per animal was between 3 and 10 × 10⁶. A significant main effect of the drug was detected using analysis of variance (P<.001). The Bonferroni multiple comparison test revealed that neutrophil receptor detection in the 2 and 10 hours, without drug groups was significantly increased compared with the 2 and 10 hours, with drug groups (P<.05) (Figure 1).

SKIN INFILTRATION NEUTROPHIL COUNTS

Neutrophil infiltration was significantly reduced in the treated vs untreated 2-hour ischemic groups (2 hours, with drug vs 2 hours, without drug; P<.05) and the 10-hour ischemic groups (10 hours, with drug vs 10 hours, without drug; P<.01) (Figure 2).

COMMENT

An in vivo correlation between systemic neutrophil receptor regulation and 5-LO inhibition was demonstrated. The detection of receptors on systemic neutrophils...
vitro. CR3 is expressed at a higher density and is probably distinct from those involved in activation of the respiratory burst. Activating mAbs against LFA-1 and gpl150/95, not CR3, triggers the respiratory burst in human neutrophils. Blood samples were obtained by TCP (≥3 mL) and subjected to dextran precipitation followed by a Ficoll-Hypaque gradient separation for removal of mononuclear cells and hypotonic lysis to eliminate contaminating erythrocytes. Cells were used within 2 hours to maintain greater than 98% viability.

PERIPHERAL BLOOD NEUTROPHIL ANTIBODY ASSAY

Neutrophil Preparation

Neutrophils were isolated from whole blood samples to more than 95% purity using procedures previously described for human neutrophils. Blood samples were obtained by TCP (≥3 mL) and subjected to dextran precipitation followed by a Ficoll-Hypaque gradient separation for removal of mononuclear cells and hypotonic lysis to eliminate contaminating erythrocytes. Cells were used within 2 hours to maintain greater than 98% viability.

Measurement of Antibody Binding to Neutrophils

Neutrophils were incubated with the R15.7 monoclonal antibody (mAb; Boeringher Ingelheim Pharmaceuticals, Ridgefield, Conn) for 15 minutes at 4°C, followed by washing and addition of fluorescein isothiocyanate–labeled anti-mouse IgG (Jackson Laboratories, Westgrove, Pa) for 15 minutes. Subsequently, the neutrophils were washed, resuspended in virus-free phosphate-buffered saline solution, and fixed with 2% paraformaldehyde. Binding was assessed by measuring cell-associated fluorescence on an FACS scan 2 (Becton-Dickinson, Franklin Lakes, NJ) and was analyzed using the lysis II program.

In preliminary experiments, we determined that the optimal concentration of 160 to 320 µg/mL of the R15.7 antibody caused maximal binding. Pretreatment of the neutrophils with phorbol myristate acetate enhanced binding, consistent with the expected increase in CD18 expression under these conditions (data not shown).

Reagents

The reagents included phorbol myristate acetate, ficoll-paque, dextran, sodium citrate, citric acid (Sigma Chemical Co, St Louis, Mo), diatrizoate (Winthrop Pharmaceuticals, Des Plaines, Ill), Dulbecco phosphate-buffered saline solution (Flow Laboratories, Costa Mesa, Calif), fluorescein isothiocyanate–labeled anti-mouse IgG (Jackson Laboratories), and the mAb R15.7, a mouse monoclonal against CD18 (Boeringher Ingelheim Pharmaceuticals). Previous studies have demonstrated that this antibody recognizes CD18 in guinea pig leukocytes. Results presented in this article represent mean neutrophil fluorescence of antibody-treated cells. Minimal background fluorescence was noted when neutrophils were treated with secondary antibody in the absence of the anti-CD18 antibody. The reported detectable fluorescence using the anti-CD18 antibody was reduced in proportion to the background fluorescence.

SKIN INFILTRATION NEUTROPHIL COUNTS

Biotin and streptavidin secondary antibodies with myeloperoxidase primary antibodies (Dako, Carpinteria, Calif; concentration, 1:100) were applied to paraffin-embedded tissue samples. The biotin and streptavidin were applied sequentially, followed by chromogen (3,3'-diaminobenzidine [DAB]; Sigma Chemical Co, St Louis, Mo), diatrizoate (Winthrop Pharmaceuticals, Des Plaines, Ill), Dulbecco phosphate-buffered saline solution (Flow Laboratories, Costa Mesa, Calif), fluorescein isothiocyanate–labeled anti-mouse IgG (Jackson Laboratories), and the mAb R15.7, a mouse monoclonal against CD18 (Boeringher Ingelheim Pharmaceuticals). Previous studies have demonstrated that this antibody recognizes CD18 in guinea pig leukocytes.

Forty randomly selected fields were counted at ×40 magnification. Only extravascular neutrophils were counted. The count was performed by looking at keratin and then continuing down through the dermis.

Our mAb recognized CD18, the β subunit that is shared by the major integrins: LFA-1 (CD11a/CD18), CR3 (CD11b/CD18), and gpl150/95 (CD11c/CD18). This group of integrins is responsible for endothelial cell interactions and activation of the respiratory burst. However, the signals involved in adhesion and spreading are likely distinct from those involved in activation of the respiratory burst. Activating mAbs against LFA-1 and gpl150/95, not CR3, triggers the respiratory burst in vitro. CR3 is expressed at a higher density and is probably responsible for interactions with intercellular adhesion molecules, including adhesion and spreading, and, unlike LFA-1 and gpl150/95, CR3 is up-regulated with activation. In vitro studies link intrinsic 5-LO activity to up-regulation of CR3, and in vivo studies show that CR3-blocking mAbs attenuate reperfusion injury in the liver and in ischemic gastric mucosa.

The main known products of 5-LO metabolism are the leukotrienes, and they are powerful paracrine mediators of inflammation. The leukotrienes, especially leuko-

![Figure 1. Mean CD18 neutrophil receptor detection with SE bars.](image-url)
triene B₄, are released into the systemic circulation and may be involved in systemic neutrophil priming and receptor up-regulation by enhancing the activity of phospholipase A₂ and increasing substrate (arachidonic acid) for 5-LO.¹⁹ Results of in vitro studies show that zileuton use inhibits neutrophil adherence and chemotaxis, indicating that intrinsic neutrophil 5-LO activity is required for these rate-limiting interactions with ischemic endothelial cells.¹ These functions are not restored by adding exogenous leukotriene B₄, suggesting an additional, previously unidentified, intracellular signaling role for 5-LO products.¹

Systemic administration of a 5-LO inhibitor is effective in reducing i/r injury in flap tissue, most likely because of an attenuated interaction between the systemic neutrophil and the ischemic flap endothelium. There is a significant reduction in neutrophil receptor expression with 5-LO, reducing the priming of systemic neutrophils from substances released into the circulation after flap reperfusion (circulating cytokines).¹⁰ We have begun to study other known products of 5-LO metabolism to determine whether they may have a role in CR3 up-regulation.

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