

# The Role of Xanthine Oxidase and Xanthine Dehydrogenase in Skin Ischemia<sup>1</sup>

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The importance of sequential events which lead to skin necrosis has significant implications in trauma, vascular injury, and wound healing. In this series of experiments, we tested the hypothesis that xanthine oxidase (XO) activity was increased along an ischemic gradient of a skin flap and that the XO enzyme activity correlated with an increase in neutrophils. There were two animal groups in which the skin flaps were raised and assayed at 0, 1, or 6 hr. In the other group, they were created as bipedicle flaps for 7 days, before the distal attachment was divided and the tissue assayed. In the acutely raised flaps, some animals were treated with the XO inhibitor, allopurinol. Xanthine dehydrogenase (XD) and XO activity was measured with a fluorometric pterin assay and neutrophil concentration was measured using a myeloperoxidase marker. In this model, there was consistent skin necrosis in the distal end of the skin flap ( $48 \pm 8\%$ ). The data showed that both XD and XO activity in the distal ends was statistically significantly increased over the sham control or proximal ends of the skin flaps at 1 hr ( $P < 0.05$ ). XO activity remained elevated in the distal ends at 6 hr. Allopurinol significantly reduced the neutrophil concentrations in the distal ends of the skin flaps when compared to untreated animals ( $P < 0.05$ ). Moreover, allopurinol reduced skin necrosis to  $12 \pm 1\%$ . Preconditioning of the skin flap reduced the XO activity to sham control levels. The observations implicate XO activity as source of free radical injury in skin necrosis seen in random skin flaps. The data suggest that allopurinol may have therapeutic benefit in reducing skin necrosis. © 1994 Academic Press, Inc.

## INTRODUCTION

A better understanding of the factors responsible for skin necrosis has significant implications in trauma, vas-

cular injury, and wound healing. During onset of skin inflammation, there is the release of histamine, the generation of oxidants, and the recruitment of neutrophils which contribute to injury. Of particular importance is the role of the oxidoreductase xanthine oxidase (XO), which forms superoxide anion. The observation that allopurinol, an XO inhibitor, reduces free radical injury and enhances skin survival [1], suggesting that the formation of superoxide ion is a toxic event during skin ischemia. Studies by Im *et al.* [2] have shown increased XO activity in rat skin flaps during skin ischemia due to the conversion of xanthine dehydrogenase (XD) to XO.

Our co-workers [3] have noted that histamine present in the burn wound can directly enhance XO activity in the absence of XD conversion to XO. XO activity in endothelial cells [4] increases when they are exposed to activated neutrophils due to the conversion of XD. These observations suggest that early inflammation contributes to increased tissue XO activity during ischemia.

The regulatory mechanisms which produce increased XO activity and the free radical burst may have clinical significance since they can provide clues for strategies to reduce oxidant stress in skin flaps. The clinical phenomenon that delayed skin flaps have enhanced survival may be related to a reduction in XO activity. This hypothesis is supported by our observations that after preconditioning of rat skin flaps there is a reduction in skin oxidants and enhanced skin flap survival [5].

In this series of experiments, a flap model of skin ischemia was used to study oxidoreductase activity of XD and XO along an ischemic gradient. These enzyme activities measured during early ischemia provide evidence that there is increased XO activity during early ischemia. This correlates with the appearance of neutrophils in the ischemic end of the skin flaps.

## MATERIALS AND METHODS

### Materials

Unless otherwise stated, histochemicals and reagents were purchased from Sigma Chemical Corp. (St. Louis, MO).

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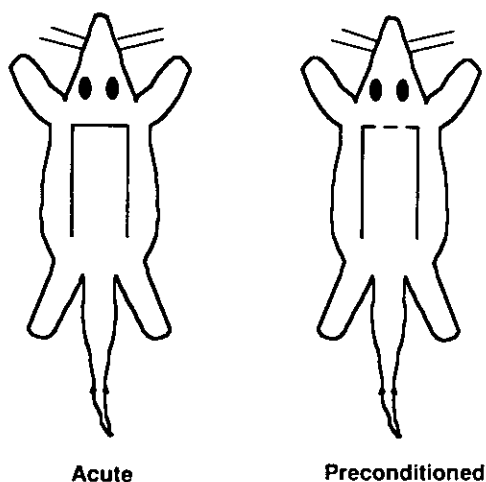


FIG. 1. The rat icons demonstrate the surgical model used to study XO and XD activity in the skin flaps. The acute flaps (left) had the distal attachment divided immediately and studies performed at various times in the subsequent 24 hr. The preconditioned skin flaps (right) were raised as bipedicle skin flaps and the distal attachment was divided 7 days later as is noted by the dashed line. After the distal attachment was divided, the skin flaps were assayed during the subsequent 24 hr.

#### Animal Model of Skin Ischemia

Sprague-Dawley rats (150–200 g) (Charles River Laboratories, Portage, MI) were anesthetized with intramuscular ketamine hydrochloride (100 mg/kg body wt; Parke Davis, Morris Plains, NJ). The rat's dorsal hair was clipped (Oster, Inc) and a 10 × 4-cm clear template was used to outline the area of study on the back. The skin flaps were outlined with a surgical marker and cut as rectangles with the base at the iliac crest using the technique of McFarlane *et al.* [5] as previously described [6, 7] (Fig. 1). The flaps were secured with surgical staples (Richard Allen Medical Inc, Richland, MI).

To "precondition" the skin, bipedicle 10 × 4-cm flaps were created on the rat's back so that the proximal and distal attachments were preserved. Seven days later, the distal attachment was transected, the flap was raised, and samples collected at various times thereafter (Fig. 1). All experiments were done under sterile conditions using 1% Betadine to prepare the skin.

To collect tissue specimens, the skin flaps were excised and placed on a T-shaped cutting board, and 0.0012-in. skin grafts were obtained from the flaps with an electric dermatome (Zimmer, Warsaw, IN). The split-thickness skin grafts were cooled to 4°C and tissue specimens for enzyme assays were obtained from the proximal and distal ends of the grafts.

All experiments were done in accord with the standards in "The Guide for the Care and Use of Laboratory Animals," DHEW Pub. No. (NIH)78/23, and were supervised by veterinarians from the Unit for Laboratory Animal Care of the University of Michigan Medical

School. The animal protocol was approved through the Animal Care Committees of both the University of Michigan and the Ann Arbor Veterans Medical Center.

#### Studies of Flap Necrosis

In separate animal studies ( $n = 5$ ), the mean amount of skin necrosis was determined at 7 days to verify that it was a reproducible model of injury. Necrosis was defined grossly by typical signs of tissue injury including black color, dehydration, eschar formation, or absence of bleeding. The total skin area and skin necrosis area were traced on transparent X-ray film for measurement. Three separate measurements of skin necrosis were obtained and the mean value in centimeters selected. The amount of skin necrosis in each group was expressed as the mean and standard deviation of the mean.

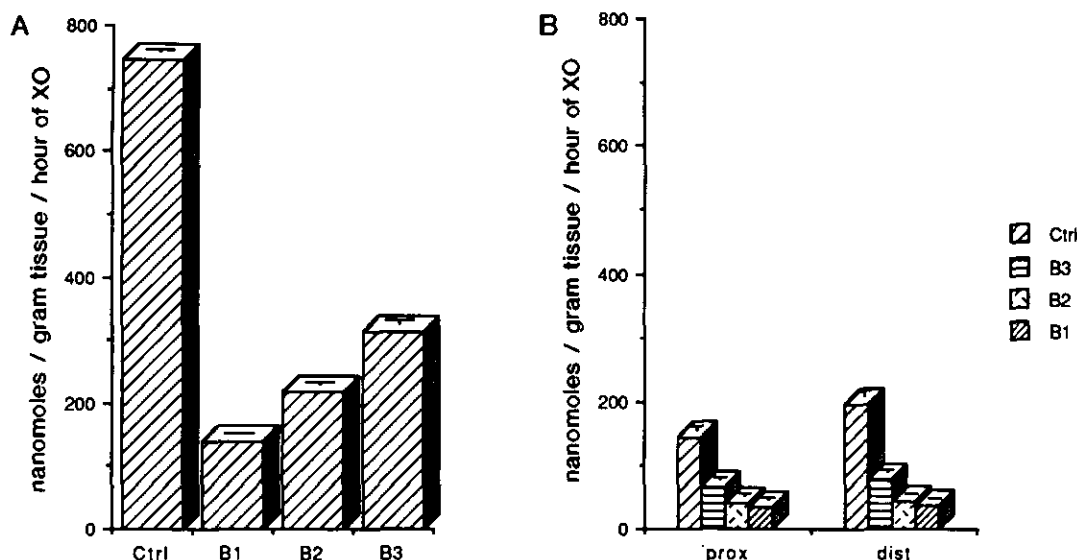
#### Animal Groups

In one group ( $n = 15$ ), acute skin flaps were raised and studied at 0, 1, or 6 hr after they were created. In a second group ( $n = 15$ ) animals were pretreated with allopurinol (20 mg/kg; Parke Davis) per oral gavage every 12 hr prior to sacrifice. In a third group ( $n = 15$ ), flaps were preconditioned (delayed) as bipedicle skin flaps for 7 days before the distal attachment was divided. Thereafter, specimens were collected at 0, 1, or 6 hr. In the four groups ( $n = 18$ ), acute flaps were raised and studied at 0, 1, 2, 3, 4, or 6 hr. In the fifth group ( $n = 6$ ), animals were treated with allopurinol as above for 7 days and skin necrosis was measured. Preliminary experiments to validate the XO assay in tissue were performed on 10 acute flaps assayed for XO activity at 1 hr.

#### Xanthine Oxidase and Dehydrogenase Assay

Split-thickness skin samples frozen with liquid nitrogen were sectioned on a cryostat (Tissue Tek; Miles Labs) into 0.011-in. sections (−2°C). Tissue sections (approximately 5 mg) are added to cold 50 mM Tris buffer, 1 mM phenylmethylsulfonyl fluoride, pH 8.1, and mixed with a vortex for 1 min. The suspension was centrifuged at 30,000g at 4°C for 30 min and the skin homogenate was frozen at −70°C until used for enzyme assays.

Xanthine oxidase activity was assayed using the fluorometric method of Anner *et al.* [2] modified for skin using 2-amino-4-hydroxypteridine (pterin) as the substrate. Enzyme activities were determined by measuring the amount of 2-amino-4,7-dihydroxypteridine (isoxanthopterin) formed in the presence or absence of methylene blue. The reaction mixture for the assay of XO activity consisted of 50 mM Tris buffer, pH 7.8, 20 μM pterin, and 1 mM EDTA. For XD activity, the reaction mixture also contained methylene blue (10 μM) and total activity (XD + XO) was determined. The XD activity was calculated by subtracting the XO activity from the total activity.



**FIG. 2.** (A) The XO activity in skin homogenates was spiked with 0.002 units of milk xanthine oxidase in 1-hr samples obtained from ischemic skin flaps. Dilutions of B103U used to inhibit XO activity were as follows: B1, 0.01 mg; B2, 0.001 mg; B3, 0.0001 mg. Note the dose-responsive reduction in milk xanthine oxidase activity in the skin homogenates after the addition of B103U. (B) The nascent XO activity in skin homogenates was assayed in skin samples obtained from flaps ischemic for one hour. XO activity was measured in the proximal (prox) and the distal (dist) ends of the flaps. Note that nascent XO activity was reduced in a dose-responsive fashion with the addition of various concentrations of inhibitor B103U to the skin homogenates. Dilutions of B103U used to inhibit nascent XO activity were as follows: B1, 0.01 mg; B2, 0.001 mg; B3, 0.0001 mg.

Twenty microliters of the skin homogenate (10–20  $\mu$ g protein) was incubated with 100  $\mu$ l of the reagent mixture for 30 min at 3°C. The reaction was stopped with the addition of 2 ml of 50 mM acetate buffer, pH 5.3. The fluorescence of the isoxanthopterin produced was measured in a fluorometer (SLM 8000; SLM Instruments) with excitation at 345 nm and an emission of 390 nm. Standards with isoxanthopterin (50–100 pmole) and reagent blanks were measured simultaneously with the samples. Allopurinol (10  $\mu$ M) was added to some reaction mixtures for an endogenous peroxidase control. Triplicate samples were employed for each assay and enzyme activity was expressed as nmole/g tissue/hr.

In some experiments, bovine milk xanthine oxidase (0.002 units) was used as a positive control. A specific inhibitor, B103U,<sup>2</sup> was used to inhibit XO activity [9] in skin homogenates.

#### Myeloperoxidase Assay

Neutrophil concentration in the tissue was measured in skin grafts with a myeloperoxidase activity assay using a modification of the technique of Bradley and Priebe [10]. Wet tissue (0.300 g) was frozen in liquid nitrogen and suspended in 6 ml of 100 mM sodium phosphate, pH 6.0, with hexadecyltrimethylammonium bromide (0.167 mg/ml) and 5 mM EDTA. The specimens were homogenized (2 min) with a Polytron instrument

(Brinkman Instruments Inc., Westburg, NY) and sonicated (Kontes, Vineland, NJ) at a setting of 5 for a multiple of three 10-sec pulses at 5°C and centrifuged (30 min, 40,000g).

Triplicate aliquots (50  $\mu$ l) of the skin homogenate were brought to a volume of 1.5 ml with assay buffer containing potassium phosphate (100 mM, pH 6), hydrogen peroxide (0.3%), and *o*-dianisidine hydrochloride (1.0%). The absorbance was read at 460 nm (Gilford, Response II) after 3 min of incubation at 37°C and the data expressed as absorbance per gram of tissue (abs/g).

#### Data Analysis

Data were compared using a paired Student *t* test while specimens were obtained from the same animals within each group. Results were expressed as the mean and standard deviation of the mean. All of these comparisons were made using assays of tissue obtained from the proximal and distal ends of the skin flaps. Comparison between different animals within the same group were made using one-way analysis of variance. Statistical significance was defined at a *P* < 0.05.

## RESULTS

The purpose of the experiments was to survey activity for XO and XD in flap skin. When the skin flaps were raised on a rat's back and examined at 7 days, there was reproducible skin necrosis ( $4.7 \pm 0.8$  cm) in the distal halves of the flap (*n* = 5). There was no skin necrosis

<sup>2</sup> Generous gift from Dr. Thomas Spector (Wellcome Research Laboratories, Research Triangle Park, NC).

**TABLE 1**  
**The XO and XD Activity (nmol/g/hr) in Skin Grafts ( $n = 3$ ) Taken from Skin Flaps at 0, 1, and 6 hr**

	0 hr		1 hr		6 hr	
	P	D	P	D	P	D
XD	60 ± 11	58 ± 3.5	49 ± 6.0	77 ± 9.0	95 ± 3.3	79 ± 5.4
XO	187 ± 20	201 ± 18	175 ± 6.8	253 ± 25*	169 ± 3.3	206 ± 2.3*

Note. Samples were obtained from the proximal (P) and distal ends (D). The data are expressed as the mean and standard deviation from the mean. Each assay was performed in triplicate on three animals for each data point. Significance was defined as  $P < 0.05$ .

\*  $P < 0.05$  compared to the proximal skin flap using a paired Student  $t$  test.

when the flaps were preconditioned for a week and the distal attachment was divided. These observations formed the basis for the study of XO and XD activity in the proximal and distal end of the skin flaps.

To insure that the XO enzyme assay was unaffected by inhibitors, dose-response curves were established from skin homogenates obtained from ischemic tissue. These homogenates were spiked with commercially available milk xanthine oxidase and measured with the pterin assay (Fig. 2A). The data demonstrated that milk xanthine oxidase activity was detected in the spiked skin homogenates and was reduced in a dose-dependent manner with the specific inhibitor of xanthine oxidase, B103U. We concluded from these experiments that milk xanthine oxidase activity could be measured in tissue homogenates and was responsive to the specific inhibitor B103U. To prove that the pterin assay measured nascent XO activity present in skin flaps, B103U was added to ischemic skin homogenates and reduced XO activity to sham control levels (Fig. 2B). These observations validated our tissue assay of XO activity which was used in subsequent studies of skin ischemia.

Using this assay, nascent XO activity control rat tissue homogenates contained  $130 \pm 4.0$  nmole/g tissue/hr. Significant XO activity ( $171 \pm 9.0$  nmole/g tissue/hr)

could be produced with local skin injections of the known inflammatory agent, phorbol myristate acetate ( $P < 0.05$ ).

To study the effect of early ischemia on the increases in XD and XO activities, enzyme activities were measured at 0, 1, and 6 hr (Table 1). During the sampling period, the XD activity in the distal ends of the skin flaps peaked at 1 hr and was statistically significantly increased in the distal ends of the sham controls and 1-hr samples ( $P < 0.05$ ). At 6 hr, XD activity increased significantly in the proximal flap, but did not increase in the distal flap. There was a significant increase in XO activity which peaked at 1 hr in the distal flap. XO activity was statistically significantly higher than that at either of the distal ends of the sham controls. The data suggests that there was a gradient increase in XO activity toward the more ischemic end since XO activity was statistically significantly higher than that in the proximal end.

To explore the possibility that XO activity could be blocked with the specific inhibitor allopurinol we pretreated the rats and studied XO activity in the flaps at 1 and 6 hr (Table 2). The data demonstrates that allopur-

**TABLE 2**

**XO Activity (nmol/g/hr) in Rat Skin Flaps Treated with Allopurinol (20 mg/kg) po Compared to Untreated Controls**

	Control		Treatment	
	PROX ± SD	DIST ± SD	PROX ± SD	DIST ± SD
0 hr	187 ± 20	201 ± 18	117.3 ± 6.8	122.0 ± 11.9
1 hr	175 ± 6.8	253 ± 25	89.3 ± 7.6†	87.4 ± 11.2*
6 hr	169 ± 3.3	206 ± 2.3	67.5 ± 6.8†	76.3 ± 7.5*

Note. The data show that allopurinol-treated animals did not develop increases in XO activity. Data are expressed as the mean and standard deviation of the mean.

†  $P < 0.05$  compared to proximal end.

\*  $P < 0.05$  compared to distal end.

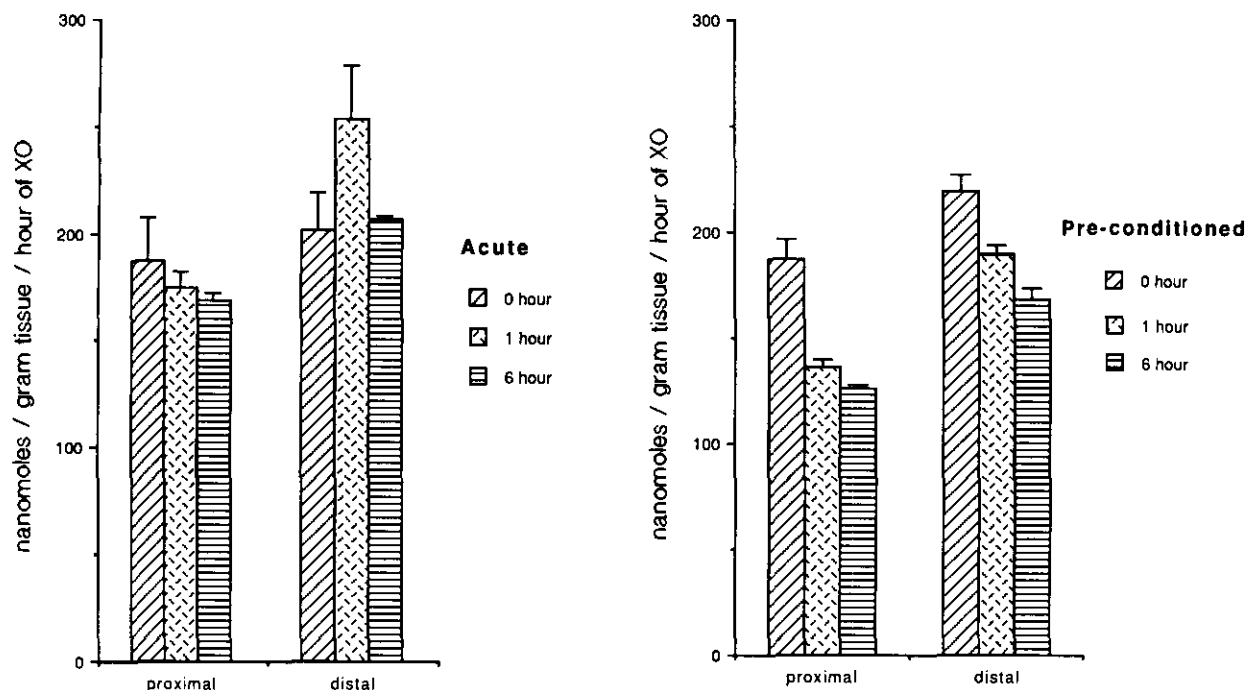
**TABLE 3**

**Myeloperoxidase Activity Expressed as abs/g Taken from Flaps at the Proximal or Distal End of the Flap**

Control		Treatment	
PROX ± SD	DIST ± SD	PROX ± SD	DIST ± SD
0.6281 ± 0.132	0.6198 ± 0.0084	0.6095 ± 0.0095	0.6298 ± 0.0112
0.6688 ± 0.0048	0.7856 ± 0.0139	0.647 ± 0.0045	0.7078 ± 0.0054
0.6764 ± 0.0412	0.8238 ± 0.0268	0.6843 ± 0.0021	0.7313 ± 0.0276*
0.664 ± 0.0094	0.8776 ± 0.0477	0.6654 ± 0.0332	0.7670 ± 0.0312*
0.6965 ± 0.0183	0.9432 ± 0.0499	0.6534 ± 0.0166	0.8292 ± 0.0399*
0.6989 ± 0.035	1.0095 ± 0.0719	0.6800 ± 0.0287	0.8459 ± 0.065*

Note. Animals were pretreated with allopurinol and compared to the no-treatment group using ANOVA. Three animals were used for each data point. Data are expressed as the mean and standard deviation from the mean.

\* Significance was defined as  $P < 0.05$ .



**FIG. 3.** (Left) The data shows an increase in XO activity at 1 hr compared to the proximal end of the acute skin flaps that was statistically significant ( $P < 0.05$ ). The XO activity in the proximal end of the flaps remained constant throughout the 6-hr period of observation. (Right) The data shows that the XO activity in preconditioned flaps was significantly reduced at 1 and 6 hr compared to acute flaps (left). Note that there was no increase in XO activity at 1 hr in the preconditioned flaps.

inol was very efficient at abolishing all XO activity in ischemic skin and completely eliminated the increased XO activity at 1 hr.

Table 3 addresses the possibility that the XO spike was associated with neutrophil recruitment in the flaps. The data showed that there was an increase in MPO activity in the distal ends of the skin flaps after 1 hr. When the XO activity was blocked with allopurinol, there was a reduction in the MPO activity in the distal skin flaps for 6 hr compared to untreated controls ( $P < 0.05$ ). The data suggested that increased XO activity contributed to neutrophil recruitment.

Since the data suggested that increased XO activity contributed to neutrophil recruitment, we blocked the XO activity with allopurinol and tested for skin necrosis in acute flaps 7 days later. These experiments demonstrated a reduction of necrosis to  $12 \pm 1.0\%$  which was statistically significantly lower than that in untreated acute flaps ( $P < 0.05$ ).

Since preconditioning of the skin flaps prevent skin necrosis, we tested the hypothesis that XO or XD activity was reduced in these flaps. There was no significant increase in XD activity (data not shown). Figure 3 compares the XO activity in acute and preconditioned flaps where there was significant reduction in XO activity when compared to sham controls on proximal ends of the flaps at 1 or 6 hr ( $P < 0.05$ ). Moreover, XO activity in the distal skin flaps did not rise above sham control levels once the distal attachment was divided.

## DISCUSSION

Critical to defining the mechanism of ischemic skin flap necrosis is the determination the sequential events leading to injury. In random skin flaps [11], there is a gradient reduction in blood as distance from the base of the flap increases. Since the predominate blood supply of the human skin is from cutaneous perforators, there is a great similarity between human skin and the rat random skin flap. This study surveyed the role of the oxidoreductase activity of XO and XD in early ischemic skin and their effect on the recruitment of neutrophils. Our data demonstrated that both XD and XO activities were highest in the distal ends of skin flaps where vascular perfusion is least. In these flaps, the XO activity peaked at 6 hr and fell gradually over 24 hr. These observations imply that maximal superoxide anion production occurs early after the initiation of skin ischemia. Our data also suggest that local tissue factors such as histamine may enhance XO activity because cromylin sodium reduces this effect.

Our previous studies [6, 7] suggest that neutrophils are actively recruited to the ischemic end of skin flaps. We propose that the rapid rise in XO activity may increase free radical production and initiate cellular membrane injury which enhances the neutrophil chemotactic activity for progressive neutrophil migration towards the greatest XO activity. This hypothesis is supported by the observation that allopurinol blocks XO activity,

reduces neutrophil concentrations, and prevents skin necrosis. Ward and co-workers [unpublished] have noted a reduction in neutrophil recruitment with PMA-stimulated skin that was pretreated with allopurinol. Other authors [12, 13] have reported that pretreatment with allopurinol reduced skin necrosis in rats and pigs. These observations taken together suggest that XO activity plays an active role in skin injury.

The importance of observations concerning the preconditioning effect is that this mechanism is linked to skin flap survival. Our previous studies suggest that neutrophils play a prominent role in skin necrosis since they are not present in preconditioned skin [6, 7]. Studies by Im have shown increases in the glycolytic enzymatic pathway, which may reduce available substrate for these oxidoreductases during ischemia [14]. Preliminary data from our laboratory suggest that the antioxidant enzymes glutathione reductase [15] and DT diaphorase (unpublished) are increased after skin preconditioning. Clearly, these studies of the preconditioning effect may provide insight to methods for enhanced survival of skin grafts, flaps, or tissue transplantation.

Although superoxide anion generated from xanthine oxidase activity may produce tissue injury, neutrophil recruitment potentiates this effect through potent metalloproteinases, elastases, and collagenases to augment oxidant effects in the wound [16]. Neutrophils also combine halide ion with hydrogen peroxide to form hypochlorous acid, another potent oxidant that injures tissue.

These studies provide a clinical rationale for blocking XO activity during tissue ischemia which may enhance tissue survival by reducing neutrophil recruitment. We have identified increases in human XO activity in human muscle and small bowel after reperfusion during microsurgical reconstruction. These observations suggest that human tissue responds to ischemia in a fashion similar to that of our animal model and supports clinical protocols to reduce XO activity during periods of ischemia [17]. Hopefully, these studies will provide impetus for further work in this area.

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