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Original Contribution

LUNG INJURY AND COMPLEMENT ACTIVATION: ROLE OF NEUTROPHILS AND XANTHINE OXIDASE

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Abstract—Evidence is presented that oxygen products generated from xanthine oxidase (XO) may also be involved in the pathogenesis of neutrophil-mediate lung injury following intravascular activation of complement with cobra venom factor (CVF). CVF injection in rats resulted in a rapid increase in plasma of both XO activity (but not xanthine dehydrogenase) and its reaction product, uric acid. These changes were greatly attenuated in allopurinol-treated animals. The appearance of XO activity was paralleled by a rise in plasma of histamine. Prevention of histamine release by pretreatment of rats with cromolyn abolished both the rise in plasma histamine and the increase in XO activity. Since we have previously shown that histamine can enhance XO activity in vitro and in vivo (Am. J. Pathol. 135:203, 1989), these observations suggest that the increase in plasma XO activity following CVF injection is related to the appearance in plasma of histamine. Accordingly, pretreatment of rats with xanthine oxidase inhibitors (allopurinol, lodoxamide) or prevention of histamine release by pretreatment with cromolyn significantly attenuated development of lung injury following injection of CVF. Our data support the concept that oxygen radicals derived from both neutrophils and XO are playing a role in the CVF-induced acute lung injury.

Keywords—Xanthine oxidase, Histamine, Oxygen radicals, Neutrophils, Complement, Lung injury, Rats, Free radicals

INTRODUCTION

Activation of the complement system in rats following intravascular injection of cobra (Naja naja) venom factor (CVF) leads to acute lung injury in which pulmonary capillary endothelial cells are the chief target of injury. Development of the lung microvascular injury is neutrophil-dependent,[1] requires activation of C5,[2] and is accompanied by the appearance in plasma and lung tissues of lipid peroxidation products.[3] Both lung injury and lipid peroxide generation are significantly attenuated by pretreatment of experimental animals with catalase, hydroxyl radical scavengers or iron chelators.[3] In vitro studies demonstrated that neutrophil-mediated killing of pulmonary artery endothelial cells is also largely prevented by catalase, iron chelators, or hydroxyl radical scavengers[4] and that the target cells are the source of the iron requirement.[5] In an isolated perfused rat lung model, similar data have been obtained.[6] These observations suggest that activation of blood neutrophils and their generation of an iron-catalyzed conversion product of hydrogen peroxide, most likely the hydroxyl radical, are directly responsible for injury of lung vascular endothelial cells.

Oxygen radicals may originate from different sources including enzyme systems of mitochondria and endoplasmic reticulum, the cyclooxygenase pathway, NADPH oxidase, and xanthine oxidase.[7–10] While the NADPH oxidase appears to be the major source of oxygen radicals in cell and tissue injury during acute inflammatory reactions involving phagocytic cells,[11] xanthine oxidase appears to play an important role in experimental ischemia-reperfusion injuries.[12–14] Following reoxygenation of ischemic tissues, superoxide anion may be generated by the enzyme xanthine oxidase (XO) which is assumed to be activated during ischemia by a calcium-triggered limited proteolysis of xanthine dehydrogenase (XD).[15] In rat pulmonary artery endothelial cells (RPAEC), conversion of XD to XO has been observed following in vitro contact of RPAEC with activated neutrophils[16] or exposure of RPAEC to human recombinant C5a or tumor necrosis factor.[17] Furthermore, recent observations suggest that physiologic concentrations of histamine can enhance XO activity in vitro as well as in vivo.[18]

In vitro experiments involving the killing of rat pulmonary artery endothelial cells, as assessed by release

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of ^{51}Cr, have indicated that both oxygen products from phorbol ester activated neutrophils as well as products of xanthine oxidase in endothelial cells are related to endothelial cell damage.[16] Furthermore, the product of XO appears to be O_2^-,since preloading of endothelial cells with superoxide dismutase greatly attenuated their killing by activated neutrophils.[19]

In order to determine if XO activity and histamine contribute to CVF-induced acute lung injury, we have examined CVF-treated rats for the appearance of XO activity and histamine in plasma and evaluated the effects on CVF-induced pulmonary injury of interventional measures including XO inhibitors, histamine receptor antagonists, and the histamine release blocker, cromolyn.

MATERIALS AND METHODS

Animal model of lung injury

As recently described,[1] specific pathogen-free, adult (250–350 g), male Long Evans rats were used for the studies to be presented. Twenty units of cobra venom factor (CVF) per kg body weight were injected intravenously along with an aliquot of ^{125}I-bovine serum albumin (^{125}I-BSA). Animals were anesthetized with ketamine hydrochloride (100 mg/kg) (Parke Davis and Co., Morris Plains, NJ) and exsanguinated 30 min later via the vena cava posterior. The lung vasculature was then perfused through the right cardiac ventricle with 10 mL phosphate-buffered saline (pH 7.4). The lungs were removed and the amount of radioactivity remaining within the tissue assessed with a gamma counter. Lung damage was defined by the increased lung vascular permeability, as determined by the ratio of radioactivity present within total lung tissue and the amount of radioactivity present in 1.0 mL venous blood obtained when the animals were killed. To test for the effects of XO inhibitors on CVF-induced lung injury, allopurinol (50 mg/kg body weight) and lodoxamide (5 mg/kg) (Upjohn Co., Kalamazoo, MI) were employed. Allopurinol was given intraperitoneally 20 min prior to CVF. Lodoxamide was injected intravenously 5 min before CFV treatment. Diphenhydramine (H1 antagonist) and cimetidine (H2 antagonist) were dissolved in 1.0 mL saline and injected intraperitoneally (50 mg/kg) 5 min before CVF. Cromolyn, dissolved in 1.0 mL saline, was injected intravenously (20 mg/kg) 15 min prior to CVF. Protection from lung injury was determined by the following equation:

$$\text{Protection (\%)} = 100 \times \left[1 - \frac{\text{Test value} - \text{Saline value}}{\text{Positive value} - \text{Saline value}}\right]$$

Sample preparation and storage

Blood samples were drawn from the posterior vena cava. The artificial conversion of xanthine dehydrogenase to xanthine oxidase was minimized by rapid sample processing in the presence of dithiothreitol (DTT) and a protease inhibitor (phenylmethyl sulfonyl fluoride, PMSF). Blood samples were therefore collected into a processing and storage medium containing potassium phosphate (0.0024 M) - NaCl (0.15 M) buffer, pH 7.35; DTT (10 mM); PMSF (1 mM); and EDTA (10 mM). Samples were continuously processed such that no more than 2 h occurred between collection and analysis. Blood samples for histamine analysis were collected in cold plastic tubes containing EDTA (10 mM) and then cooled immediately on ice. All samples were centrifuged at 4°C within 10 min following the sample collection. Following the recommendations of Lorenz et al,[20] only the upper part of plasma was used for immediate histamine determination.

Xanthine dehydrogenase/xanthine oxidase activity. Uric acid formation.

XD and XO activities were assayed by measurement of uric acid formation at 293 nm in the presence or absence of NAD^+ at 37°C, as described by Waud and Rajagopalan.[21] The reaction mixture contained 100 μL xanthine (50 μM), 100 μL NAD^+ (500 μM) or buffer in the absence of NAD^+, 600 μL potassium phosphate (0.0024 M) and sodium chloride (0.15 M) at pH 7.35, and 100 μL rat plasma to a final plasma content of 5% (v/v). Furthermore, as indicated, the reaction mixture contained 100 μL of the uricase inhibitor 2,4 dihydroxy-6-carboxy-1,3,5 triazine (oxonate)[22] (Aldrich Chemical Company Inc., Milwaukee, WI) present in a final concentration of 3 μM. XO and XD activities were expressed as [nmol uric acid formed/ml of plasma/min].

Uric acid determination

Plasma levels of uric acid were determined spectrophotometrically at 293 nm and expressed as [μM] using a molar extinction coefficient of 7.59 $cm^{-1}mM^{-1}$ for uric acid.[23] Proof that uric acid was being measured was obtained by the addition of uricase.

Histamine radioimmunoassay

To determine plasma histamine we used a commercially available kit from Amak Inc. (Westbrook, ME). This assay is based on the acylation of plasma histamine and its competition with ^{125}I-acylated histamine for binding to a mouse monoclonal antibody. Results were calculated employing a standard curve from seven standard samples ranging between 0 and 150 nM. Results were expressed as [nM].

Table 1. Effect of various interventional measures on CVF-induced acute lung injury

Treatment	Number of Animals	Lung Injury* (x ± SEM)	Significance (p value)	Protection (%)
Saline	5	0.17 ± 0.02		
CVF (20 U/kg)	5	1.04 ± 0.11		
CVF plus				
Allopurinol (50 mg/kg)	4	0.49 ± 0.04	<0.01**	63
Lodoxamide (5 mg/kg)	6	0.60 ± 0.07	<0.01**	51
Cromolyn (20 mg/kg)	4	0.31 ± 0.12	<0.01**	84
Diphenhydramine (50 mg/kg)	4	0.85 ± 0.14	NS**	22
Cimetidine (50 mg/kg)	4	0.53 ± 0.12	<0.05**	59

*Permeability ratio (Calculations are described in Methods Section). **Compared to CVF values.

Materials

Unless otherwise stated, reagents were purchased from Sigma Chemical Corp. (St. Louis, MO) and were of the highest purity available.

Statistical analysis

Data in various groups were expressed as mean ± standard error of the mean (SEM). For comparing of the response between two treatments a paired t test was used. To determine the significance of differences between controls and multiple experimental groups two-way analysis of variance in combination with the Dunnetts multiple comparisons test was used. Statistical significance was defined as $p < 0.05$. Specifics regarding the number of experiments, and so forth, are contained in the text or within Table 1.

RESULTS

Increases in plasma xanthine oxidase activity following CVF injection

Intravenous bolus injection into rats of cobra venom factor (CVF) resulted in a rapid increase in plasma XO activity (defined as in vitro production of uric acid in the presence of oxonate) for the first 10 min post CVF application, followed by a fall in the levels of plasma XO (Fig. 1). This increase in enzyme activity was restricted to XO activity; no rise in xanthine dehydrogenase (XD) was found after injection of CVF (Fig. 1). In

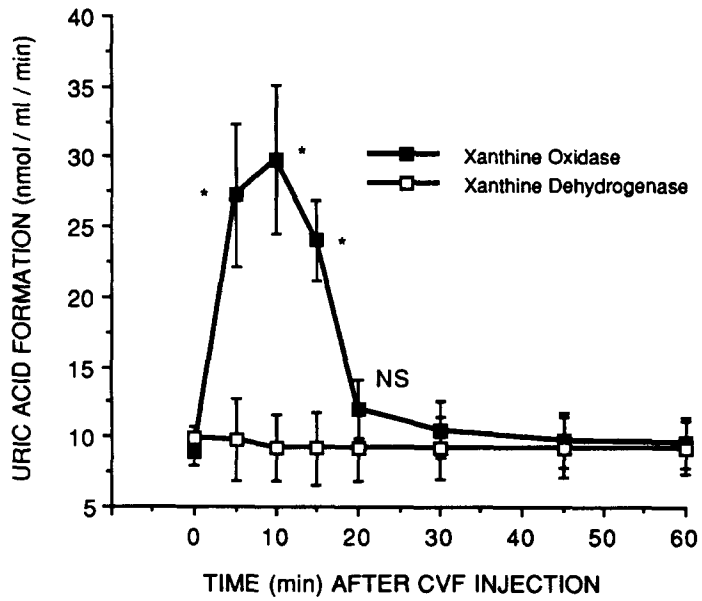

Fig. 1. Rat plasma levels of xanthine oxidase and xanthine dehydrogenase following systemic complement activation by intravenous injection of cobra venom factor (CVF; 20 U/kg). *p<0.01 compared to time-zero value; NS: not significant; four to six animals were examined at each time point.

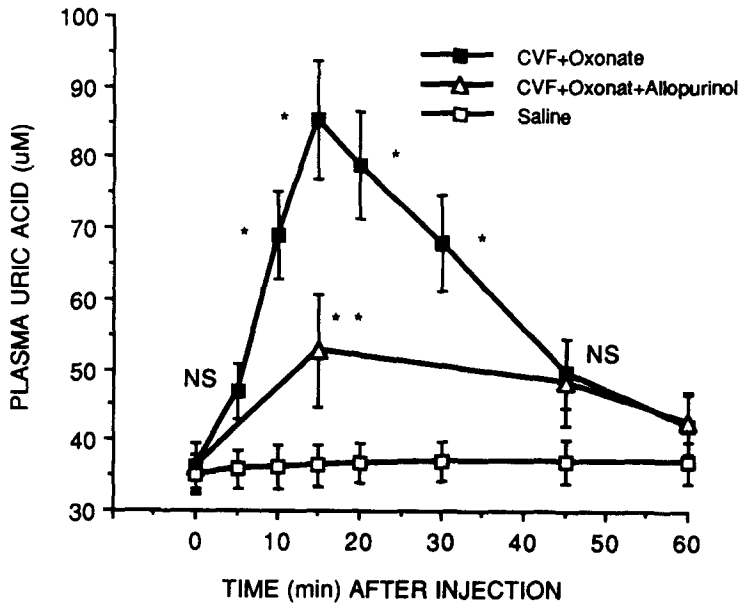

Fig. 2. Appearance of uric acid in plasma of rats injected with cobra venom factor (CVF; 20 U/kg). Maximal increases of uric acid were seen when rats were pretreated with the uricase inhibitor, oxonate (50 mg/kg; i.p.), which prevents the conversion of uric acid to allantoin. Pretreatment with the xanthine oxidase inhibitor, allopurinol (50 mg/kg; i.p.) significantly reduced plasma levels of uric acid. *$p<0.01$ compared to time-zero value; **$p<0.05$ compared to 15-min value (CVF+oxonate); NS: not significant; each data point represents the mean obtained from three to five animals.

order to demonstrate if uric acid production occurs in vivo following CVF injection, animals were pretreated with oxonate (50 mg/kg; i.p.) in order to prevent uricase-mediated breakdown of uric acid to allantoin. The rise in plasma XO activity (Fig. 1) was accompanied by a significant increase in plasma levels of uric acid peaking 15 min after CVF injection (Fig. 2). Pretreatment of CVF-injected rats with allopurinol (together with oxonate) significantly diminished the plasma levels of uric acid (Fig. 2). These observations suggest that systemic complement activation following CVF injection results in both increased plasma levels of XO activity and its reaction product uric acid (and, consequently, superoxide and hydrogen peroxide).

CVF-induced increases in plasma histamine levels and their effect on xanthine oxidase activity

Recently, we have shown in vitro that histamine can enhance the catalytic activity of XO (but not XD) and have demonstrated in vivo that acute dermal microvascular injury (edema) in rats following thermal trauma is mediated by oxygen radicals generated by histamine-enhanced XO.[18] In order to examine if a similar mechanism of histamine-dependent augmentation of XO may play a role in CVF-induced acute lung injury, plasma histamine levels were determined and the effect of cromolyn treatment on the activity of plasma XO following CVF injection was examined. As shown in Fig. 3A, intravenous injection of CVF in rats caused a rapid increase in plasma histamine levels, peaking at 10 min post CVF, essentially paralleling the changes in plasma of XO activity (Fig. 3B). Treatment of experimental animals with the membrane stabilizer, cromolyn, completely prevented the appearance of histamine in plasma of CVF-treated rats (Fig. 3A). Furthermore, the suppression of histamine release by cromolyn also completely prevented the increase in plasma of XO activity (Fig. 3B). The addition in vitro of histamine (1 μM) to plasma obtained 15 min after CVF injection into rats that were pretreated with cromolyn caused an increase in XO activity from 5.7 ± 0.23 nmol/ml/min ($n=3$) to 17.03 ± 0.44 nmol/ml/min ($n=3$) similar to that shown in Fig. 3B. These observations suggest that histamine being released in the course of CVF-induced systemic complement activation is playing a crucial role in modulating the catalytic activity of plasma XO activity.

Protection from acute lung injury

In the model of acute lung injury accompanying systemic activation of complement induced by infusion of CVF, the resulting injury is known to be related to the generation of H_2O_2 by activated neutrophils and demonstrates a requirement for iron, suggesting a possible role for ·OH.[24] If XO-derived oxygen radicals play a role as well, both treatment of experimental animals with inhibitors of XO or the prevention of histamine release by

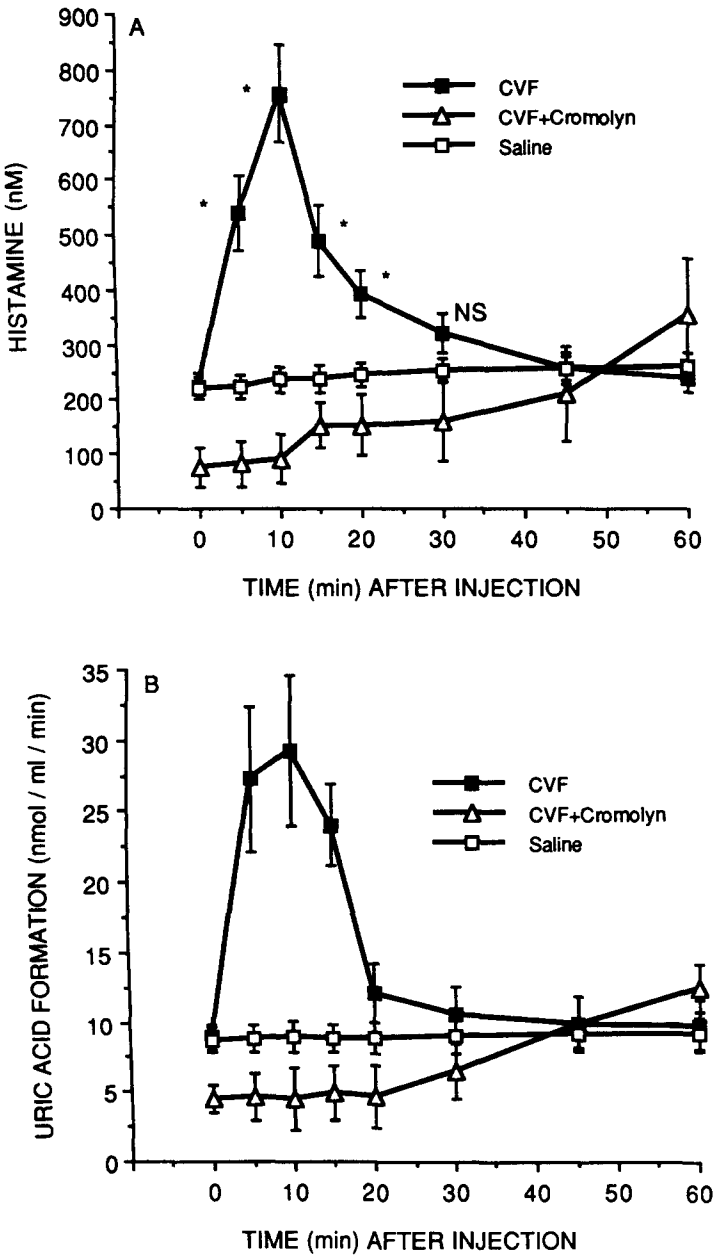

Fig. 3. Concomitant appearance of histamine (Frame A) and xanthine oxidase activity (Frame B) in plasma of rats injected with cobra venom factor (CVF; 20 U/kg). Pretreatment of experimental animals with cromolyn (20 mg/kg; i.v.) prevented release of histamine (Frame A) thus resulting in loss of histamine-mediated enhancement of plasma xanthine oxidase activity (Frame B). Histamine and XO activity were measured in the same animal. *$p<0.01$ compared to time-zero value; NS: not significant; four to six animals were used at each time point and treatment.

cromolyn (see above) should attenuate acute pulmonary injury in the rat following CVF injection. Accordingly, the experiments as shown in Table 1 were accomplished. Intravenous injection of CVF into rats produced a six-fold increase in lung vascular permeability (to 1.04 ± 0.11) as compared to saline-infused animals (0.17 ± 0.02). Pretreatment of animals with the XO inhibitors, lodoxamide[25] or allopurinol, resulted in a 51% and 63% reduction in CVF-induced lung injury, respectively ($p < 0.01$). A striking attenuation of acute lung injury was also observed when animals were treated with cromolyn (Table 1). These observations support the concept that histamine may play a significant role in complement-dependent and oxygen radical-mediated acute lung injury by enhancing plasma XO activity in vivo.

Surprisingly, the histamine H2 receptor antagonist, cimetidine, also attentuated the lung injury, whereas the H1 receptor antagonist, diphenhydramine, had no sig-

nificant effect (Table 1). Whether stimulation of H2 receptors plays a role in CVF-induced pulmonary injury remains unclear. However, the protective effect of cimetidine may as well be explained by its known ability to effectively scavenge hydroxyl radicals, whereas diphenhydramine possesses little scavenging capacity.[18]

DISCUSSION

In earlier studies, we demonstrated that systemic complement activation by cobra venom factor (CVF) results in acute lung microvascular injury and that this injury depends on the availability of neutrophils and their production of toxic oxygen metabolites.[1,23] This was supported by the observation that neutrophil depletion of experimental animals prior to intravascular complement activation or pretreatment with antioxidant enzymes, iron chelators, or hydroxyl radical scavengers resulted in significant protection from pulmonary injury. Morphologic studies have revealed damage of lung alveolar capillary endothelial cells in areas of intimate contact with neutrophils and, adjacent to these areas, destruction of vascular basement membrane, fibrin deposition, interstitial and intraalveolar edema, and intraalveolar hemorrhage. The trigger for this event is intravascular complement activation and generation of $C5a^2$ which causes activation and sequestration of blood neutrophils in the lung vasculature and their release of oxygen radicals. Endothelial cell-derived oxidants may augment this event.[26] Because interventional measures using antioxidant enzymes, iron chelators, or hydroxyl radical scavengers consistently provided higher levels of protection than did neutrophil depletion, [1,23] additional source(s) of toxic oxygen metabolites had to be considered. The present observations point at xanthine oxidase (XO) as being the additional source of oxygen radicals involved in the pathogenesis of acute lung injury following systemic complement activation.

The data obtained in the present study demonstrate that, when intravascular activation of complement occurs, the resulting acute lung injury can be attenuated by XO inhibitors, suggesting involvement of XO-generated oxygen radicals in the tissue injury. In addition, there is evidence of increased plasma levels of both XO and uric acid following CVF-induced complement activation. This increase is significantly attenuated by administration of the XO inhibitor, allopurinol, as demonstrated in the experimental animals. Since neutrophil depletion, XO inhibitors, and antioxidants are protective, it can be assumed that both neutrophil and XO-derived oxygen radicals are playing a role in the CVF-induced acute lung injury.

As indicated above, this pattern is consistent with what we now believe to be the mechanism by which activated neutrophils kill rat pulmonary artery endothelial cells. Presumably, H_2O_2 production by the neutrophils and O_2^- generation by endothelial cells (via XO) results either in formation of ·OH via an iron-dependent pathway, or H_2O_2 could conceivably cause nitric oxide (NO·) generation in the endothelial cells, resulting in an interaction of NO· with O_2^- to generate ·OH via an iron-independent pathway.[27]

The rise in plasma XO activity was found to be paralleled by significant increases in plasma histamine levels. Prevention of histamine release by pretreatment of CVF-injected rats with cromolyn also prevented the increase in plasma XO activity and significantly attenuated the oxidant-mediated lung injury. This is in accordance with recent studies from our laboratory which provided evidence to suggest that histamine plays a crucial role in the modulation of XO activity following thermal injury of rat skin. In this model, increased levels of XO activity appear in plasma and oxidants derived from XO have been shown to be largely responsible for the microvascular injury (edema formation) in the burned skin. Friedl et al.[18] have observed in vitro that histamine and its metabolic derivatives increase the catalytic activity of XO (but not XD) in rat plasma and rat pulmonary artery endothelial cells. Histamine also enhanced the activity of purified XO obtained from human milk. Following thermal injury of rats, it was then observed that the increase in plasma XO was paralleled by a significant rise in plasma histamine levels, whereas the plasma XD levels remained completely unchanged.[18] As in the CVF lung injury model, the rise in both plasma histamine and XO activity could be prevented by cromolyn pretreatment of the thermally injured rats. These interventional measures also attenuated the development of thermal skin edema.[18] Taken together, these observations suggest that the enhancement by histamine of the catalytic activity of XO may be of general importance in the pathogenesis of inflammatory reactions. The mechanism by which histamine can enhance the catalytic activity of XO is not yet known.

Following systemic complement activation in rats, plasma levels of XO activity and concomitant uric acid production reach peak values within 10 to 15 min of CVF injection. Full development of oxidant-mediated acute lung injury can be observed within 20 to 30 min post CVF. Although some of the oxygen radicals are being released from activated blood neutrophils sequestered in the lung microvasculature, our data support the concept that toxic oxygen species derived from plasma XO are also participating in the lung microvascular injury. This hypothesis of endothelial cell injury being brought about by oxidants derived from circulating XO is supported by recent reports in the literature. Parks et al.[28] reported that intraarterial infusion of XO into nonischemic intestines produced an increase in microvascular injury that was comparable to that ob-

served after ischemia/reperfusion and was significantly attenuated by antioxidant interventions. Accordingly, XO activity (in amounts that were released into the perfusate of ischemic livers), when supplied with substrate, was shown to produce severe vascular endothelial injury in vitro, even in the presence of serum or whole blood.[29]

Generation of C5a following systemic complement activation has been shown to be essential for the development of lung microvascular injury after CVF injection.[2] In this model, C5a appears to be important for both the massive accumulation of blood neutrophils in the lung vasculature and the liberation of histamine from mast cells and basophils. As mentioned above, histamine plays an important role because of its ability to significantly enhance XO activity and thus directly affect oxidant production by this enzyme. Prevention of histamine release by cromolyn completely prevented the increase in plasma XO activity and reduced lung injury by 84%. The observation that cromolyn is more protective than the XO inhibitors (51–63% protection) is somewhat surprising but may be explained, in part, by its mildly inhibitory effect on neutrophil accumulation.[30] There is also the possibility that histamine per se may cause some increase in lung microvascular permeability following systemic complement activation. This would explain why the H1 antagonist, diphenhydramine, exhibited a 20% (although nonsignficant) reduction in lung injury as determined by the extravasation of ^{125}I-BSA. The lung protective effect (59%) of the H2 antagonist, cimetidine, may be explained by the fact that it is an effective scavenger of hydroxyl radical.[18]

The protective effects of allopurinol and lodoxamide have to be viewed with some caution since these XO inhibitors have also been found to scavenge hydroxyl radical.[18,31] Since hydroxyl radical has been identified as the most likely oxidant involved in CVF-induced lung injury,[24] and because of the fact that rather high doses of allopurinol had to be used in vivo (up to 50 mg/kg body weight), it seems possible that protection from CVF lung injury by allopurinol is at least partially related to scavenging of hydroxyl radical. However, it can be calculated that the likely plasma level of lodoxamide, which (at 5 mg/kg) was similar protective, is in the low μM-range, too low to allow for significant ·OH scavenging. Because the generation of superoxide anion, hydrogen peroxide, and uric acid is the result of the action of XO on its substrates, hypoxanthine and xanthine, the inhibitory effects of the XO inhibitors on uric acid production and lung tissue injury further support our assumption that the CVF-induced lung microvascular injury is, at least in part, being mediated by XO-generated oxygen radicals.

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ABBREVIATIONS

CVF—cobra venom factor
XD—xanthine dehydrogenase
XO—xanthine oxidase