American Journal of Pathology, Vol. 134, No. 6, June 1989

Xanthine Oxidase Activity in Rat Pulmonary Artery Endothelial Cells and Its Alteration by Activated Neutrophils

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The possibility that endothelial cell-derived oxidants could contribute to neutrophil-mediated endothelial cell injury and cytotoxicity has been a subject of speculation. Rat pulmonary artery endothelial cells (RPAECs) were examined for the presence of xanthine oxidase (XO) activity, a well-known source of O_2^-. Using a sensitive assay based on measurements of radioactive xanthine conversion to uric acid by high performance liquid chromatography (HPLC), RPAEC extracts were found to contain both XO and xanthine dehydrogenase (XD) activities. Extracts from early passage cells have 55.3 ± 11.7 (mean ± SE) units/10^6 cells of total (XO + XD) activity, one unit of activity being defined as the conversion of 1% of substrate to product in 30 minutes of incubation. XO comprised 31.6 ± 3.1% of this total activity. Addition of human neutrophils stimulated with phorbol myristate acetate (PMA) caused a rapid and dose-dependent increase in RPAEC XO activity from 31.6 ± 3.1% to 71.7 ± 4.8% of total without altering total (XO + XD) activity. The neutrophil dose-response curve for increase in XO paralleled closely the curve for neutrophil-mediated RPAEC cytotoxicity. The basal XO and XD activities and the neutrophil-induced increase in XO activity were inhibited by treating RPAECs with allopurinol, oxypurinol, and lodoxamide, which also inhibited cytotoxicity, but not by catalase, superoxide dismutase, or deferoxamine. Addition of H_2O_2 failed to cause an increase in RPAEC XO activity or XD to XO conversion. The results suggest that during neutrophil-mediated injury, rapid conversion of RPAEC XD to XO occurs, resulting in increased XO-catalyzed endogenous oxidant production, which may contribute to the oxidant burden in the killing mechanism initiated by activated neutrophils. Although the mechanism for conversion of XD to XO is uncertain, it appears that neutrophil-derived H_2O_2 is not sufficient to cause this phenomenon. Furthermore, neither O_2^- nor chelatable iron is required for neutrophil-induced XD to XO conversion. Supernatant fluids from activated neutrophils failed to induce XD to XO conversion in RPAECs. This* in vitro *system provides an opportunity to define the cellular and molecular mechanisms underlying the* in vivo *phenomenon of XD to XO conversion associated with ischemic/reperfusion or inflammatory tissue injury. (Am J Pathol 1989, 134:1201–1211)

Neutrophil-mediated injury to endothelial cells is thought to be important in many forms of tissue injury.[1,2] The mechanism of this injury is considered to be related to toxic products such as oxidants, as well as to proteases secreted by stimulated neutrophils. Recent data, however, suggest that the endothelial cell is not a passive cell or target and is actually an important source of inflammatory and immune mediators.[3–11] Unlike the neutrophil, the endothelial cell has not been shown to contain NADPH (Nicotinamide adenine dinucleotide phosphate, reduced form) oxidase, the O_2^--generating enzyme in the neutrophil. However, there is recent evidence to suggest that chelatable iron in the endothelial cell is critical for the process of injury initiated by activated neutrophils.[12] This observation, in conjunction with the recent finding that xanthine oxidase (XO) in the presence of substrate and iron

Supported by National Institutes of Health grants HL28737, HL31963, GM28499, and GM29507 (University of Michigan); and HL21568 and HL33064 (University of Miami). Part of this work was performed during the tenure of an Established Investigatorship (SHP) from the American Heart Association.

Accepted for publication February 20, 1989.

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can produce a reactive oxidant distinguishable from hydroxyl radical,[13] implies that endothelial cell XO could play a key role in neutrophil-mediated injury. The presence of XO in endothelial cells has been suggested by indirect studies using XO inhibitors and by immunologic techniques using specific anti-XO antibodies.[14,15] The latter study indicates the presence of XO in significant quantities in capillary but not large vessel endothelial cells.[15] More recently XO activity has been demonstrated in isolated bovine endothelial cells.[16]

XO in the presence of xanthine reduces O_2 to O_2^-.[17] Other potent oxidants have also been reported as by-products of this reaction.[13,18] In most tissues this enzyme is usually present predominantly in the dehydrogenase form, which uses NAD^+ (Nicotinamide adenine dinucleotide) instead of O_2 as the electron acceptor.[19] Conversion from xanthine dehydrogenase (XD) to XO has been observed under a variety of nonphysiologic as well as physiologic conditions. For instance, isolation of milk XO under nonreducing conditions results in most of the activity being recovered as the oxidase, part of which reverts to the XD form on addition of sulfhydryl reducing agents.[20] Similar situations exist in assays of tissue extracts wherein rapid conversion of XD to XO occurs in the absence of reducing agents.[21,22] Irreversible conversion of XD to XO also can occur by proteolysis,[20,23] possibly by calcium-dependent proteases.[24-26] The actual mechanisms responsible for *in vivo* conversion of XD to XO are not clear, although conditions of ischemia followed by reperfusion appear to be associated with the conversion process.[22,26,27] An increase in XO activity could represent an important source of oxidants involved in the injury process. This possibility is supported by studies showing that certain types of ischemia-reperfusion injury can be ameliorated by allopurinol, oxypurinol, and other XO inhibitors.[14,28-30]

The current study was undertaken to examine if XO and XD activities are present in rat pulmonary artery endothelial cells (RPAEC) and whether XD to XO conversion occurs during neutrophil-mediated injury. The role of these enzyme activities in neutrophil-mediated endothelial cell cytotoxicity was then examined by comparing dose-response curves and examining the effects of XO inhibitors. Evidence is also presented, suggesting that the killing of endothelial cells by activated neutrophils *in vitro* involves a pathway in which xanthine oxidase plays a role.

Materials and Methods

Reagents

Hypoxanthine, xanthine, uric acid, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol, superoxide dismutase, Triton X-100, Ficoll-Hypaque (Histopaque), bovine serum albumin (BSA), buttermilk XO, and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co., (St. Louis, MO). Bovine milk XO (1.32 U/mg protein) was purchased from Calbiochem (La Jolla, CA). [8-^{14}C]-xanthine (40 to 60 mCi/mmol) was purchased from ICN Biomedicals, Inc. (Irvine, CA). Na[^{51}Cr]O_4 was obtained from New England Nuclear (Boston, MA). Sources for media and other reagents for tissue culture were as previously indicated.[12] HPLC grade methanol was obtained from Burdick & Jackson (Midland, MI). Tetrabutylammonium phosphate was purchased from Aldrich Chemicals (Milwaukee, WI). Deferoxamine mesylate (Desferal) was from Ciba-Geigy (Summit, NJ). Unless otherwise specified, all other chemicals were of reagent grade or better.

Endothelial Cells

RPAECs were isolated from the pulmonary vasculature by perfusion of microcarrier beads into the vessels and subsequent retrieval of the beads with endothelial cells attached by retrograde perfusion.[31-33] On isolation, the cells exhibited the typical cobblestone morphology of endothelial cells. They were positive for factor VIII by immunofluorescence, bound acetylated low-density lipoprotein, and had high levels of angiotensin-converting enzyme (ACE) (3.2×10^4 to 1.9×10^5) molecules/cell) as measured with the synthetic substrate ^{3}H-Benzoyl-phe-ala-pro.[34-36] The cells were maintained in monolayer culture using Ryan red medium. They were passaged by mechanical scraping using a rubber policeman without exposure to proteases. Growth was at 37 C and 5% CO_2. Stocks were kept frozen in liquid N_2. Throughout the course of the study, the endothelial cells maintained their cobblestone morphology and levels of ACE activity. For enzyme assays, cells were plated onto 35-mm wells of 6-well plates and allowed to grow to confluence overnight, resulting in approximately 1×10^6 cells per well. For cytotoxicity assays, 2×10^5 cells were plated onto 16-mm wells of 24-well plates and used as described previously.[12]

Neutrophils

Human neutrophils were isolated from peripheral blood using a slight modification of Boyum's method as described previously.[12,37] The cells were finally suspended in Hank's balanced salt solution (HBSS) containing 0.02% BSA (HBSS-BSA) and kept on ice until use.

To test the effects of neutrophil secretions on RPAEC enzyme activities, neutrophils were suspended in HBSS-BSA at 7.5×10^6 cells/ml and stimulated with either 100 ng/ml PMA or 10^{-7} M N-formylmethionylleucyl-phenylalanine plus 5 μg/ml cytochalasin B. After a 30-minute incu-

bation at 37 C, the cells were centrifuged and the neutrophil supernates collected for testing as described below.

Enzyme Assays

Confluent RPAEC monolayers containing approximately 10^6 cells per well were prepared as described above. On the day of assay, media were removed and the cells washed 3 times with HBSS-BSA. Two milliliters of this buffer was then placed in each well, except for those wells to be treated with neutrophils or neutrophil supernates, wherein 2 ml of neutrophil suspension or supernates was added. The standard treatment contained 1.5×10^7 neutrophils per 35-mm well. Where indicated, inhibitors were added to the RPAECs at this time. Final concentrations of inhibitors used were as follows: 0.1 mM allopurinol, 1 mM oxypurinol, 1 mM Iodoxamide, 100 units/ml catalase, 50 μg/ml superoxide dismutase, or 1.0 mM deferoxamine. The plates were then kept at 37 C in a CO_2 incubator for 15 minutes, following which PMA (final concentration of 100 ng/ml) was added to those wells to be stimulated by this agonist. Incubation was then allowed to proceed for another 30 minutes, except for the time-course studies as indicated in the figure legends. Media were removed, and the wells washed twice with phosphate-buffered saline (PBS). Seven hundred microliters of ice-cold extraction buffer was then added into each well. Extraction buffer contained 50 mM sodium pyrophosphate, pH 8.3, 1 mM ethylenedamine-tetraacetate (EDTA), 0.2% Triton-X100, 1 mM dithiothreitol (except where indicated otherwise), and 1 mM PMSF. After standing for 5 minutes, the buffer was repeatedly pipetted back and forth to ensure total cell lysis and extraction. Control studies comparing this method *vs.* mechanical scraping followed by sonication showed equivalent recovery of enzyme activities, thus indicating complete recovery by this extraction method.

From each sample, two 300-μl aliquots were taken and placed in two separate 12×75 mm disposable plastic tubes. NAD^+ (0.7 mM, final concentration) was added to one tube for measurement of total (XO + XD) activity, while the other tube received buffer only (50 mM sodium pyrophosphate, pH 8.3, and 1 mM EDTA) for measurement of XO activity. Radioactive substrate was prepared by diluting ^{14}C-xanthine in cold xanthine to give a final concentration of 59 μM total xanthine with a specific radioactivity of 8.5 μCi/μmol when added to each assay tube. Reaction was allowed to proceed for 30 minutes at 37 C. Using this amount of cell extract, the assay was linear for at least 60 minutes of incubation (Figure 1). The samples were then transferred to disposable ultrafiltration units (Centricon-10, Amicon Corp., Boston, MA), spiked with 30 μl of 1 mM uric acid, and centrifuged at 4 C for 45 minutes. The ultrafiltrate containing radioactive product

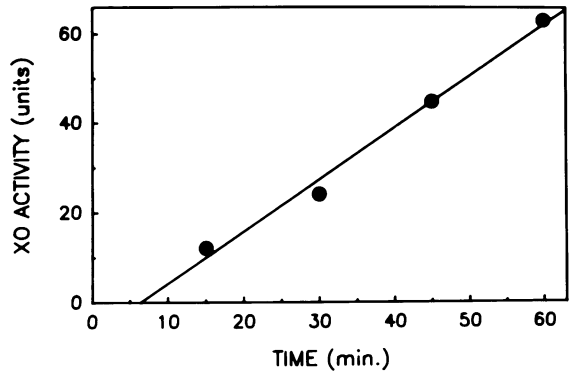

Figure 1. *Kinetics of XO assay; 300 μl of RPAEC extracts were assayed for XO activity as described in Materials and Methods. At the indicated times of incubation with substrate, the reaction was stopped and samples analyzed for uric acid formation by HPLC. Activity is in units as defined in Materials and Methods. The assay is linear for at least 60 minutes.*

(uric acid) and excess substrate was then collected and stored frozen in tightly capped vials at −20 C for storage until analysis, usually within 48 hours, by HPLC. This period of storage did not cause any artifactual alteration in the amount of detectable uric acid product.

Quantitation of the radioactive uric acid product was undertaken by reverse phase HPLC using the ion pairing reagent tetrabutylammonium phosphate. The instrumentation consisted of a Varian Instruments (Palo Alto, CA) Vista LC5560 chromatograph equipped with two detectors connected in series, a UV-visible variable wavelength detector (model UV-200, Varian Instruments), and an online radioactive detector (Flo-One, model HS, Radiomatic Instruments, Tampa, FL). A DS-654 data system (Varian Instruments) was used for peak quantitation. Programmed injection of samples at 0.01 minutes was undertaken with a Rheodyne model 7126 automatic injector using a 50 μl loop. Separation was accomplished using a Supelco (Bellefonte, PA) LC-8 column, containing C8, 3 μ stationary phase, and measuring 4.6×150 mm. Fifty microliters of ultrafiltrate prepared as described above was injected onto the column and eluted isocratically at 30 C with a flow rate of 1 ml/min. The solvent composition was 93% A, which is 5 mM tetrabutylammonium phosphate in H_2O, and 7% B, which is CH_3OH. Under these conditions, baseline separation of xanthine from uric acid was observed with retention times of 4.2 to 4.7 minutes for xanthine, and 8.7 to 11.6 minutes for uric acid. The retention time for uric acid became shorter as the number of injections increased, however, baseline separation from the xanthine peak was maintained after 50 injections. The column was exhaustively washed with CH_3OH and H_2O after every 50 samples. The radioactive uric acid peak was identified during each run by the UV (254 nm) absorbance of the uric acid standard with which the samples were spiked before ultrafiltration as described above.

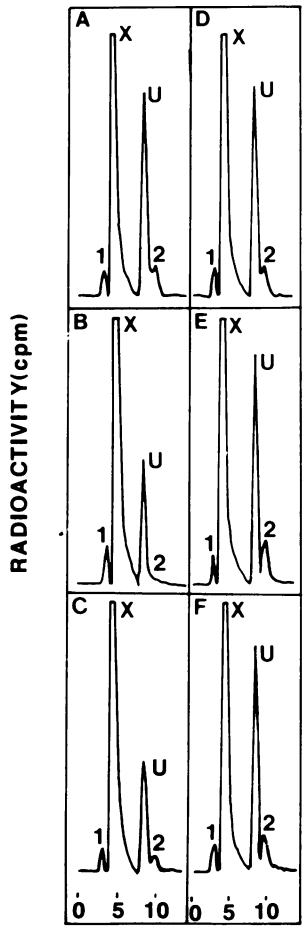

Figure 2. *Effects of activated neutrophils and H_2O_2 on RPAEC enzyme activities. RPAEC extracts were assayed for XO and XO + XD activities as described in Materials and Methods. At the end of the assay incubation, the samples were ultrafiltered and injected onto the HPLC column. Representative chromatograms representing XO (A to C) and XO + XD (D to F) activities are shown. The effects of treating RPAECs with PMN + PMA (A and D) and 0.1 mM H_2O_2 (B and E) are compared with untreated controls (C and F). Peaks labeled X and U refer to xanthine and uric acid, respectively, whereas peaks 1 and 2 correspond to an impurity present in the commercial ^{14}C-xanthine preparation, and an unidentified by-product of the XO/XD reaction, respectively. Because peak 1 did not interfere in the assay and quantitation of the results, it was not removed before assay. Enzyme activity was calculated by expressing the areas under peaks U and 2 as a percentage of the sum of the areas in peaks X, U and 2.*

This peak also was confirmed by its absence when 0.1 mM allopurinol was added to the reaction mixture. Typical chromatograms are shown in Figure 2. An impurity present in the commercial ^{14}C-xanthine preparation was identified as peak 1 in the chromatograms. Because its removal before assay did not affect the separation and quantitation, no attempt was made to do this for subsequent assays. Peak 2 was present as a minor (usually <15% of the main peak) shoulder to the main uric acid peak when high activities were encountered. This peak also was abolished by treatment with allopurinol, suggesting that it was also a product related to XO activity. Thus, calculations of activities included this peak as product. Activities were expressed as percent of total random integration units contained in the uric acid peak including peak 2, relative to the total integration units contained in both xanthine and uric acid peaks. Expression in this manner corrected for possible variation in total sample recovery from the column. One unit of activity was empirically defined as the amount of enzyme that could cause the conversion of 1% of substrate to product in 30 minutes of incubation at 37 C, a pH of 8.3, and in the presence of 59 μM substrate. This unit of activity is equivalent to 0.69 μmols of uric acid formed per minute at 37 C. The assay remained linear for activities as high as those that consumed 60% of added substrate, using either authentic XO (Calbiochem) or RPAEC cell extracts (Figure 3). Such saturation also was observed when authentic XO was added to RPAEC cell extracts. Under the above assay conditions, however, none of the endothelial cell extracts exceeded 50% substrate consumption. The assay was tested for specificity by examining the effects of known XO inhibitors on the activity of authentic XO (Calbiochem). Figure 4 shows the dose-dependent inhibitory effect of allopurinol and lodoxamide on authentic XO, thus confirming the validity of the assay. All results obtained were within this linear range.

Cytotoxicity Assay

This was measured as percent specific ^{51}Cr release from preloaded RPAECs exactly as described previously using bovine endothelial cells.[12]

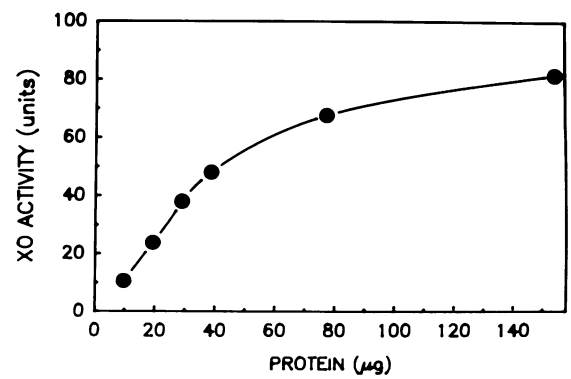

Figure 3. *Linearity of XO assay as a function of enzyme dose. Authentic XO (Calbiochem) was added at the indicated amounts, and assayed as described in Materials and Methods. Units of activity are defined in Materials and Methods. The assay is linear when less than 60 units of activity are measured (corresponding to about 60 μg of authentic XO).*

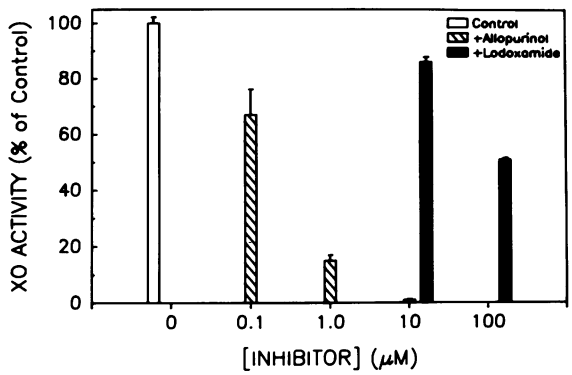

Figure 4. *Effects of XO inhibitors on XO assay. Authentic XO (20 μg) was assayed in the presence of the indicated doses of inhibitors. Both compounds inhibited authentic XO in a dose-dependent manner.*

Statistical Analysis

For comparison of mean values between two sets of data, the two-tailed Student's *t*-test was used, using the standard criterion of $P < 0.05$ to indicate statistically significant differences. Unless otherwise indicated, each experiment was repeated twice.

Results

Enzyme Activities in RPAECs

Using the assay conditions described above, both XO and XD activities were easily detectable in RPAECs, as shown in Figure 2 and Table 1. These cells were found to have 53 ± 8.57 units/10^6 cells of total XO + XD activity. The range of values obtained in six different experiments was 40 to 74 units/10^6 cells. Slightly less than a third of this was due to XO, but this increased to greater than 45% if dithiothreitol was omitted from the extraction buffer, suggesting the presence of a reversible form of XO. Significantly lower activities were found in cells passaged greater than 30 times, becoming virtually undetectable by the 40th passage. Thus, all subsequent studies were undertaken with cells that had been passaged less than 15 times. These activities were totally inhibited by 0.1 mM allopurinol added to the assay buffer. Because extraction was undertaken in the presence of dithiothreitol, EDTA, and PMSF, this distribution of activities, namely >65% XD and the remainder XO (Table 1 and Figure 5), is probably an accurate reflection of actual conditions in the endothelial cell, and not an *in vitro* oxidative and/or proteolytic artifact. The data do not rule out the presence of a reversible (by SH reducing agents) form of XO (as noted above), which is reduced to the XD form on extraction with DTT.

Table 1. *Effects of Activated Neutrophils on RPAEC XO and XD Activities**

	Control	PMN + PMA	*P* value†
XO	17.4 ± 3.65	34.6 ± 7.31	0.023
XO + XD	53.0 ± 8.57	47.3 ± 8.08	NS
%XO	31.6 ± 3.14	71.7 ± 4.76	0.0014
%XO (no DTT)‡	45.9 ± 3.44	91.7 ± 6.30	0.0058

* PMN and PMA doses are as described in Materials and Methods. Enzyme activities are defined in Materials and Methods, and expressed as mean ± SE in units per 10^6 cells, except for %XO, which refers to XO activity expressed as a percentage of the total XO + XD activity.

† Calculated using the paired Student's *t*-test comparing control *vs.* PMN + PMA mean values obtained during each experiment. These results summarize the data from four (three for %XO (no DTT)) separate experiments performed on different days. NS refers to $P < 0.05$.

‡ This refers to the omission of dithiothreitol from the cell extraction buffer.

Effects of Neutrophils

Addition of PMA-activated human peripheral blood neutrophils caused a significant increase in RPAEC XO activity without a concomitant increase in total (XO + XD) activity. Results from representative experiments are shown in Figures 2 and 5, and the overall results from four separate experiments using four different neutrophil and RPAEC preparations are summarized in Table 1. Addition of 1.5×10^7 activated neutrophils for 30 minutes caused, on the average, a doubling of XO activity (Table 1). Without a corresponding increase in total activity, this implied that addition of activated neutrophils had caused conversion of XD to XO, such that the latter now comprised most (>65%) of the cellular enzymatic activity (Table 1 and Figure 5). PMA-activated neutrophils from a patient with chronic granulomatous disease also caused conversion of similar magnitude to 68.4 ± 0.4%. Addition of PMA or nonactivated neutrophils alone failed to cause any significant alteration in cellular enzymatic activities (Figure 5). Human neutrophils were devoid of detectable XO or XD activity.

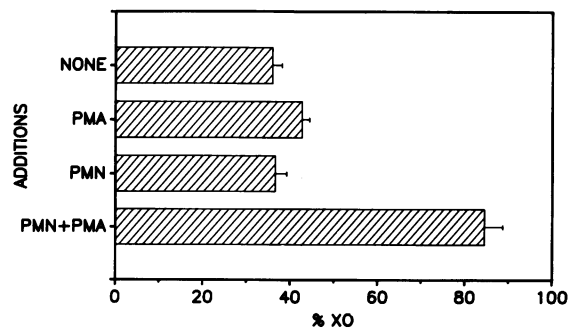

Figure 5. *Effects of PMA and PMN on RPAEC XO activity. Assay conditions and the timing of additions of 100 ng/ml of PMA and/or 1.5×10^7 neutrophils per well are described in Methods. %XO refers to XO activity expressed as a percentage of the total (XO + XD) activity, which was unaffected by all treatments. Data represent mean ± SE, with N = 3.*

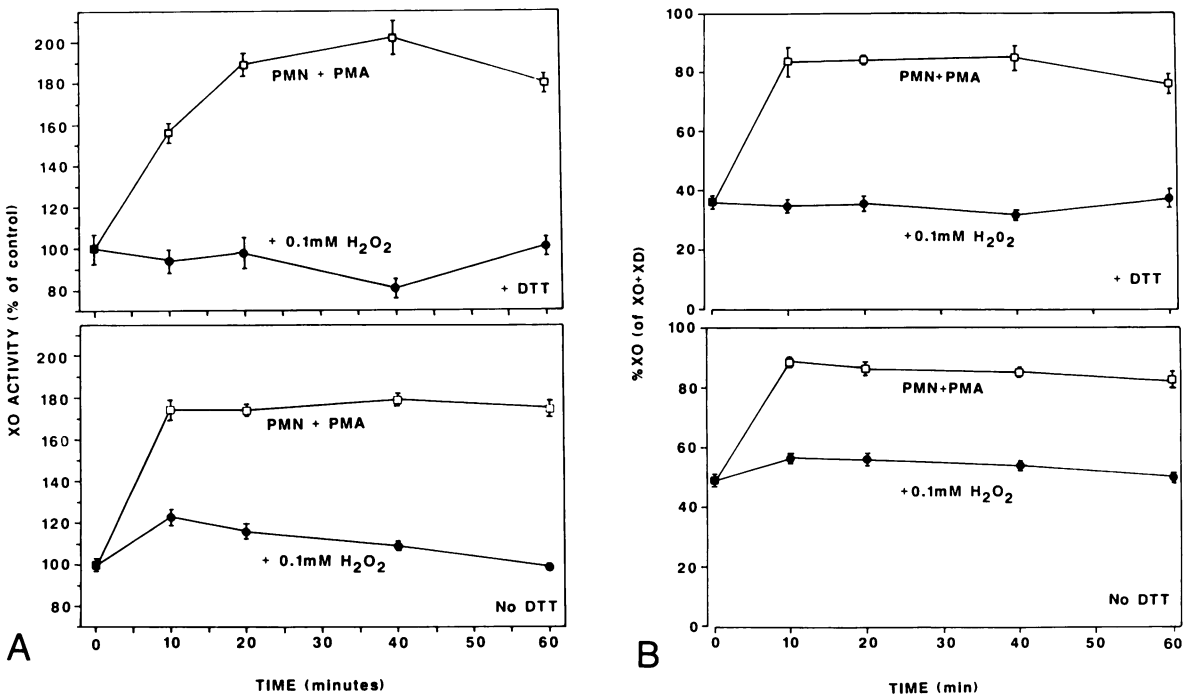

Figure 6A. *Time-course of effects on RPAEC XO activity. The effects of PMA-stimulated neutrophils (PMN + PMA) and 0.1 mM H_2O_2 on RPAEC XO activity are shown (top). Omission of dithiothreitol in the cell extraction buffer did not alter the time course or the magnitude of the effects significantly (bottom). H_2O_2 had no significant effects on XO or XD activity. XO activity is expressed as a percentage of the mean control (untreated cells) value (= 100%). Data represent mean ± SE, with N = 3. The abscissa represents time after PMA addition (for the PMN + PMA group) and after H_2O_2 addition, respectively.* **B:** *Time-course of XD to XO conversion. The ordinate represents XO activity expressed as a percentage of the total (XO + XD) activity, which was not significantly altered by all treatments. Thus, any increase in %XO is due to XD to XO conversion. Top and bottom panels refer to extraction with or without dithiothreitol, respectively. Abscissa and data are expressed as described in the legend to Figure 3.*

Because the same extent of conversion was observed even when dithiothreitol was omitted from the extraction buffer (Table 1 and Figure 6), neutrophil-induced XD to XO conversion was not reversible, at least by sulfhydryl reducing agents. Also, because proteolytic inhibitors were present in the extraction buffer, it is unlikely that the conversion occurred by proteolysis during cell disruption and preparation of samples for assay. The time course of this activated neutrophil-induced XD to XO conversion was relatively rapid, reaching essentially maximal levels 10 minutes after addition of PMA (Figure 6), which was independent of the presence or absence of dithiothreitol in the extraction buffer.

To examine if this XD to XO conversion is due to neutrophil secretions (ie, granule contents, oxidants, arachidonate metabolites, etc), the supernates of activated neutrophils were tested. Neutrophils were stimulated with either PMA or fMLP (N-formylmethionyl leucyl phenylalnine) plus cytochalasin B. Figure 7 shows that neither resting nor stimulated neutrophil supernates could cause significant XD to XO conversion in RPAECs. This conversion required the presence of intact neutrophils. Figure 7 also shows that fMLP plus cytochalasin B-stimulated neutrophils were capable of causing XD to XO conversion, albeit not to the degree caused by PMA stimulated neutrophils. Hence, neutrophil-derived secretions alone are not sufficient to cause XD to XO conversion.

The neutrophil-induced conversion process was dependent on neutrophil dose as shown in Figure 8, with significant effects seen with as few as 5×10^6 neutrophils added per well. Maximal effect was attained at doses equal to 1.5×10^7 or greater. This dose-response curve parallels closely the one for cytotoxicity. This similarity in dose-response curves suggests that this neutrophil-induced increase in endothelial cell XO may be important for the injury and/or killing process.

Effects of XO Inhibitors

Addition of known inhibitors of XO resulted in significant inhibition of both XO and total XO + XD activities in endothelial cells (Table 2), and significant protection of endothelial cells from neutrophil-mediated cytotoxicity (Table 3). In these experiments, the inhibitors were added to the RPAECs immediately before addition of neutrophils. To assay XO and XD enzyme activity, the endothelial cell monolayers were washed three times with PBS after exposure to neutrophils and before preparation of cell ex-

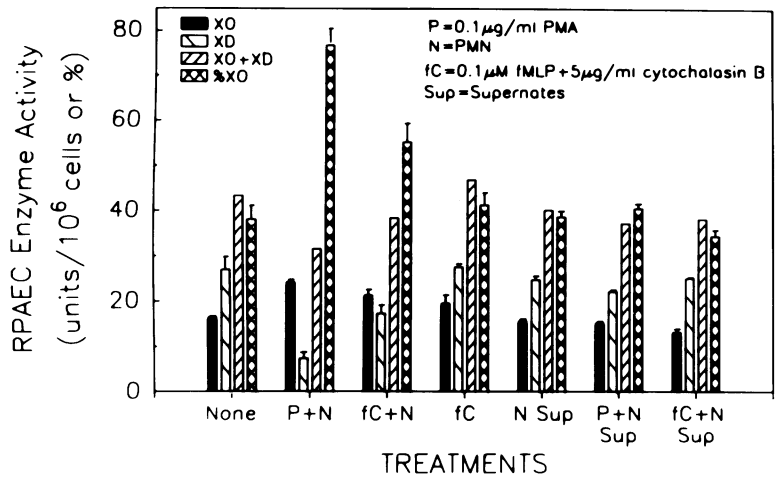

Figure 7. *Effects of neutrophil supernates on RPAEC enzyme activities. P + N and fC + N represent additions of PMA-stimulated and fMLP plus cytochalasin B-stimulated neutrophils, respectively. fC represents addition of agonists only (without neutrophils). N Sup, P + N Sup, and fC + N Sup represent addition of unstimulated, PMA-stimulated, and fMLP + cytochalasin B-stimulated neutrophils supernates, respectively.*

tracts for enzyme assay. Thus, the inhibitory effect on XO and XD activities was seen despite the removal of inhibitors from the extracellular fluid, suggesting either entry of these inhibitors into cells, or their tight association with the cells without penetration through the plasma membrane. Inhibition by allopurinol and oxypurinol was greater than 85% for all activities in both control and neutrophil-exposed cells (Table 2). Lodoxamide at comparable concentrations was less potent when incubated with intact RPAECs and more selective in its inhibitory activity, showing a greater inhibitory effect on XO than XD activity. To confirm this selectivity, endothelial cell extracts were prepared and assayed in the presence of lodoxamide added at the time of substrate addition. The results show this selective effect for XO *vs.* XD activity (Table 4) by lodoxamide at a dose of 0.1 mM (60% inhibition of XO *vs.* 31% inhibition of XD). This selectivity was not apparent at a dose of 1 mM, which caused greater than 90% inhibition of all activities (Table 4). Addition of similar doses of allopurinol and oxypurinol to the extracts in this manner caused total inhibition of all enzyme activities. The difference in potency between lodoxamide and the other two XO inhibitors also was seen when their effects on authentic enzyme were measured (Figure 4).

The differential effects of the three XO inhibitors on enzyme activity parallel their effects on cytotoxicity. Endothelial cells were protected from neutrophil-mediated cytotoxicity by all three XO inhibitors in a dose-dependent manner, but allopurinol and oxypurinol were much more potent than lodoxamide (Table 3), in agreement with their relative ability to inhibit XO (Figure 4).

Effects of H_2O_2

Because PMA-activated neutrophils are a plentiful source of H_2O_2, and catalase has significant protective effects on neutrophil-mediated endothelial cell injury,[38-40] XD to XO conversion could be induced by the addition of exogenous H_2O_2. Figures 2 and 6 show that 0.1 mM H_2O_2 failed to cause any significant alterations in RPAEC XO or total activities. Additions of lower (down to 0.001 mM) or higher (up to 10 mM) doses of H_2O_2 also failed to cause any significant change in either of these activities, except to inhibit at the higher doses (≧0.1 mM). Thus, H_2O_2 *per se* does not appear to be the cause for XD to XO conversion in RPAECs incubated with activated neutrophils.

Effects of Anti-oxidants

To explore further if neutrophil-derived oxidants are important for the conversion process, catalase and superoxide dismutase were incubated with mixtures of endothelial cells and activated neutrophils. The results indicated that

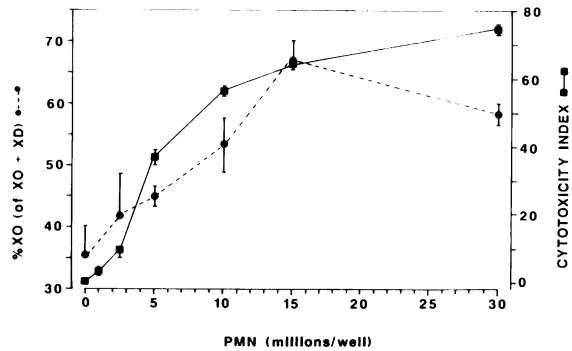

Figure 8. *Neutrophil dose-response curves for XD to XO conversion and cytotoxicity. Ordinate values are described in the legend to Figure 4 for %XO, and in Materials and Methods for cytotoxicity index. The dose-response values on the abscissa have been corrected to reflect the same neutrophil to target cell ratio for both XD to XO conversion and cytotoxicity (ie, they have been adjusted to 10^6 endothelial cells as targets in each case). Data represent mean ± SE, with N = 3 for %XO, and N = 4 for cytotoxicity.*

Table 2. *Effects of XO Inhibitors on RPAEC Enzyme Activities**

	Enzyme activity			
	XO		XO + XD	
Inhibitor	Control	PMN + PMA	Control	PMN + PMA
None	22.0 ± 1.08	46.0 ± 1.54	67.7 ± 0.55	63.1 ± 1.70
0.1 mM allopurinol	5.4 ± 0.83	4.9 ± 0.29	12.3 ± 1.02	6.4 ± 0.20
1 mM oxypurinol	1.9 ± 0.9	1.7 ± 0.86	0.7 ± 0.17	1.5 ± 0.33
1 mM lodoxamide	5.3 ± 1.47	10.9 ± 1.60	39.9 ± 3.85	26.7 ± 1.42

* Additions of PMA and PMN, and the enzyme assays are as described in Materials and Methods, except where indicated, an inhibitor was added at the indicated final concentration to the RPAECs immediately before PMN addition. Enzyme activities are defined and expressed as in Table 1. Data represent mean ± SE, with N = 3.

the addition of neither catalase nor superoxide dismutase inhibited the conversion process (Table 5), despite the ability of catalase to protect endothelial cells from neutrophil-mediated injury.[38–40] Similarly, although deferoxamine also provides protection in this type of injury,[12] it failed to inhibit XD to XO conversion (Table 5). These results confirm that neutrophil-derived oxidants, whether directly generated or iron-catalyzed, are neither sufficient nor necessary to cause RPAEC XD to XO conversion. Studies are currently underway to define the nature of the neutrophil product or function responsible for this phenomenon.

Discussion

Recent studies suggest that the endothelial cell has the potential of playing active and important roles in tissue injury. In addition to being a target of injury, it has the capability of secreting a variety of inflammatory and other mediators, some of which are harmful to itself and surrounding cells, including both O_2^- and H_2O_2.[3–11,41,42]

To determine if XO could represent a potential source of these oxidants in endothelial cells, a sensitive and reproducible assay was developed by adapting previously described methods using radioactive xanthine as substrate, and quantitation of the radioactive uric acid product.[43] RPAECs were chosen for study because of the abundance of data on models of lung injury in rats. Using this method, it was determined that RPAECs contained measurable amounts of XD and XO. Under conditions of cell extraction that were designed to minimize post-cell disruption artifacts, XO activity comprised approximately a third of the total XO + XD activity. Omission of dithiothreitol in the extraction buffer increased this ratio to approximately one half of total activity, thus suggesting the presence of a reversible form of XO in these cells. Further comparative studies need to be undertaken before this finding can be generalized to endothelial cells of other species. Species differences in the content of XO in various cells and tissues are known to occur,[19] thus making it likely that there will be differences also in the content of XO in endothelial cells from diverse species.

Neutrophil-induced endothelial cell injury may be mediated by both oxidants and proteases. The source of oxidants was originally thought to be derived solely from the activated neutrophil. Recently, however, studies using the iron chelator deferoxamine suggest that iron-dependent oxidant production by the endothelial cell also is an important component of the injury process initiated by neutrophils.[12] To examine the possibility that endothelial oxidant production may be stimulated in this injury process via increased XO activity, the effect of neutrophils on RPAEC XO activity was determined. The results show a dramatic increase in endothelial cell XO activity at the expense of

Table 3. *Effects of XO Inhibitors on Neutrophil-Mediated Cytotoxicity of RPAECs**

Inhibitors	Cytotoxicity
None	32.7 ± 1.7
1 mM Allopurinol	24.4 ± 2.4†
5 mM Allopurinol	12.8 ± 2.1†
1 mM Oxypurinol	22.3 ± 1.2†
5 mM Oxypurinol	11.9 ± 0.5†
1 mM Lodoxamide	27.8 ± 1.5
5 mM Lodoxamide	23.1 ± 1.1†

* Cytotoxicity assays were carried out in the presence or absence of XO inhibitors. Cytotoxicity is expressed as the % specific ^{51}Cr-release from preloaded RPAECs exposed to activated neutrophils and then incubated for 6 hours. Data represent mean ± SE, with N = 4.

† Indicates statistically significant difference from untreated controls (None) at $P < 0.05$.

Table 4. *Effects of Lodoxamide on Enzymatic Activities**

	Enzyme activity		
Inhibitor	XO	XD	Total (XO + XD)
None	14.8 ± 0.49	58.1 ± 2.11	72.9 ± 2.53
0.1 mM lodoxamide	5.9 ± 0.42	39.9 ± 0.55	45.7 ± 0.40
1.0 mM lodoxamide	2.7 ± 1.68	6.3 ± 2.43	9.0 ± 0.80

* Assays were done using normal untreated RPAEC extracts as described in Materials and Methods, except inhibitors at the indicated final concentrations were added immediately before addition of radioactive substrate. Activities are expressed as described in Table 1 and presented as mean ± SE, with N = 3.

XD activity when PMA-stimulated neutrophils were added, suggesting direct conversion of XD to XO. Neutrophils with impaired oxidative metabolism (from a patient with chronic granulomatous disease) also caused similar conversion. The effect was not seen with PMA or nonactivated neutrophils alone. Conversion reached maximal levels within 10 minutes of PMA addition. Such rapid rates of conversion have been reported to occur in various tissues when subjected to ischemia-reperfusion,[26,27] thus suggesting that this endothelial cell response may be physiologically relevant. Neutrophil secretions alone were not sufficient for XD to XO conversion, suggesting that direct cell-to-cell contact or communication may be necessary. The extent of conversion was dependent on the number of neutrophils present, reaching maximal values with 1.5×10^7 cells per well. This dose-response curve parallels closely that for cytotoxicity, providing support for the conclusion that XD to XO conversion represents an important mechanism for increased endothelial cell oxidant production at least in this model of neutrophil-induced injury. This conclusion is further strengthened by the ability of XO inhibitors to provide significant protection against both neutrophil-mediated (Table 3) and ischemia-reperfusion injury.[28-30,44-45] Inhibition of endothelial cell XO by these compounds may effectively cancel the damaging consequence of XD to XO conversion, although other nonspecific effects of these inhibitors cannot be ruled out at this time. Because human neutrophils do not contain XO or XD activity, and these inhibitors are able to inhibit RPAEC XO activity, the inhibitory effect of these compounds is likely to be due to the inhibition of endothelial cell XO-generated oxidants. Penetration of these compounds into the cells is likely to be enhanced by neutrophil-derived proteases and oxidants.

The mechanism of neutrophil-induced XD to XO conversion is unclear, although the results of this study indicate that neutrophil-derived oxidants and other secretory products alone are not sufficient to mediate this effect. The inability of catalase and SOD to inhibit conversion and the ineffectiveness of H_2O_2 to cause conversion, plus the ability of neutrophils from a patient with chronic granulomatous disease to cause XD to XO conversion, virtually rule out H_2O_2 and O_2^- as key mediators of the conversion process. Thus, the efficacy of catalase and deferoxamine in inhibiting neutrophil-mediated endothelial cell cytotoxicity cannot be related to their ability to inhibit XD to XO conversion. These results are consistent with the conclusion that inhibition of cytotoxicity by these agents is probably mediated by their ability to reduce overall toxic oxidant concentrations generated by both NADPH oxidase in the neutrophils and XO in the endothelial cells. Viewed from this perspective, the mechanism of cell injury in this model is primarily dependent on the overall oxidant burden, a significant portion of which is derived from increased XO activity in the endothelial cell as a result of XD to XO conversion. The importance of this endothelial cell contribution depends on the presence of this enzyme, which if variably present in different species could result in species differences in the susceptibility to this form of injury. More definitive proof would require direct quantitative analysis of the amount of oxidant production attributable to the increase in endothelial cell XO activity.

Table 5. *Effects of Anti-Oxidants on RPAEC XO Activities**

Treatment	Dose	%XO	
		Control	PMN + PMA
None	–	40.5 ± 1.83	67.1 ± 1.99
Catalase	100 μ/ml	42.1 ± 2.02	73.0 ± 0.98
SOD	50 μg/ml	42.9 ± 3.67	71.6 ± 2.89
DFO	1 mM	39.1 ± 2.16	73.8 ± 0.92

* The experimental protocol was as described in Table 3, except antioxidant enzymes or the iron chelator deferoxamine (DFO) were added at the indicated final concentrations at the time of PMN addition. Absolute enzyme activities were unaffected by addition of these agents. Data represent mean ± SE, with N = 3.

An alternative mechanism for XD to XO conversion is local hypoxia due to increased oxygen consumption by the activated neutrophils resting atop the endothelial cell monolayer. This mechanism is thought to be important in tissue and cell injury due to conditions of ischemia followed by reperfusion.[14,26,27] Under these conditions, activation of Ca^{++}-dependent proteases is postulated to result in proteolytic digestion of XD to an irreversible form of XO with lower molecular weight.[25-27] Whether this occurs in neutrophil-mediated injury remains to be determined, but the cellular system described herein provides an opportunity to test this hypothesis in a definitive and direct manner, which has not been possible with prior *in vivo* and whole tissue studies. This system can serve as a useful *in vitro* model for the previously described *in vivo* phenomenon.

Finally, the role of neutrophil granule contents in the conversion process merits commentary. The known major components that are secreted by activated neutrophils would include proteases, such as elastase, collagenase, and cathepsins, other hydrolases, and myeloperoxidase. The effects of these secretory products on endothelial cell XD to XO conversion have been negative. This would suggest a requirement for cell-to-cell contact for XD to XO conversion to occur. Further studies are needed to elucidate what aspect of neutrophil-RPAEC interaction is the critical factor or factors in causing XD to XO conversion.

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