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Short Communication

Xanthine Oxidase Potentiation of Reactive Oxygen Intermediates in Isolated Canine Peripheral Neutrophils

Dale F. Gruber, Kevin P. O'Halloran, and Ann M. Farese

Experimental Hematology Department, Armed Forces Radiobiology Research Institute, Bethesda, Maryland, U.S.A.

Summary: Oxygen-derived free radicals are believed to contribute to reperfusion injury based, in part, upon results conferred by the pharmacologic administration of allopurinol. Allopurinol inhibits xanthine oxidase (XO) activity in ischemic tissues. The possible role of XO as a pathologic mediator prompted examination of its effects on isolated peripheral canine neutrophils. In contrast to neutrophils alone, or following stimulation with phorbol myristate acetate (PMA), it was determined that XO affected both the membrane potential and the metabolism significantly. Membrane potential assay showed that at 5–10 min, PMA depolarized 89–96% of the canine neutrophils between 32–48%. Incubation with 0.5 U/ml XO involved fewer cells (54–86%), but at substantially increased cellular depolarization levels (76–90%). Metabolic assay showed that XO concentrations as low as 0.124 U induced significant cellular H_2O_2 production compared with temperature controls. At 0.25–0.5 U XO/10^6 cells, cytosolic H_2O_2 increases were almost three times those of PMA. **Key Words:** Canine—H_2O_2—Membrane depolarization—Neutrophils—Oxygen radicals—Xanthine oxidase.

Exposure of polymorphonuclear neutrophils (PMN) to soluble or insoluble stimuli, i.e., chemoattractants, bacteria, or opsonized particles, initiates a complex series of responses that may include the secretion of granules and lysosomal enzymes and the production, or release, of reactive oxygen intermediates (ROI) (1,2). It is in part by way of these specialized mechanisms that professional phagocytes are able to fulfill their bactericidal role (3). In the absence of adequate in vivo protective mechanisms, an inappropriate elaboration of ROI could result

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Address correspondence and reprint requests to Dr. D. F. Gruber at Experimental Hematology Department, Armed Forces Radiobiology Research Institute, Wisconsin Avenue, Bethesda, MD 20814-5145, U.S.A.

in lipid peroxidation and damage of normal healthy tissue (4,5). Xanthine oxidase (XO) has been reported to be one of the substrates necessary in the acellular production of reactive oxygen radicals (6–9). XO's participation in acellular generation of ROI and its presence as a normal breakdown product of tissue xanthine dehydrogenase prompted our examination of its effects on the membrane potential and metabolism, i.e., H_2O_2 production of separated canine peripheral neutrophils in comparison with changes initiated by phorbol myristate acetate (PMA), a potent stimulator of the respiratory burst in human PMNs (1).

MATERIALS AND METHODS

Reagents

The following reagents were used: PMA, xanthine oxidase, dimethylsulfoxide (Sigma Chemical Co., St. Louis, MO, U.S.A.); Hanks' balanced salt solution (HBSS), phosphate-buffered saline (PBS), and trypan blue (GIBCO, Grand Island, NY, U.S.A.); dichlorofluorescein diacetate (DCFH-DA) (Eastman Kodak Company, Rochester, NY, U.S.A.); dipentyloxocarbocyanine [DiOC5(3)] (Molecular Probes Inc., Junction City, OR, U.S.A.); ammonium chloride (Fisher Scientific, Silver Spring, MD, U.S.A.); propidium iodide (Calbiochem, La Jolla, CA, U.S.A.). Stock solutions of DCFH-DA and DiOC5(3) were stored in absolute ethanol at concentrations of 5 and 1 m*M*, respectively. PMA was dissolved in dimethylsulfoxide at 0.01 *M* and stored at −70°C. Fetal bovine serum (FBS) (Hyclone Labs, Logan, UT, U.S.A.) was heat-inactivated (56°C, 60 min) and filtered (0.45 μm) before use.

Animals

Hra beagles (*Canis familaris*), 1–2 years old weighing 10–12 kg, were used in these experiments. Canines were quarantined on arrival and screened for evidence of disease or parasitic infestation before being released for experimentation. Unless prescribed by protocol, canines were not medicated during the experiment or for 30 days before. Canines were kenneled in an AAALAC-accredited facility, provided commercial lab chow, and allowed access to tap water ad libitum. Animal holding rooms were maintained at 70 ± 2°F with 50 ± 10% relative humidity using at least 10 changes/h of 100% conditioned fresh air. Animals were maintained on a 12 hour light/dark full-spectrum photoperiod with no twilight.

Neutrophil Isolation

Peripheral blood (5 ml once a month) was drawn from the lateral saphenous vein into heparinized (10 U/ml blood) syringes. Peripheral blood was washed in HBSS without Ca^{2+} and Mg^{2+} (400 *g*, 10 min, 21°C). Contaminating red blood cells were lysed with 0.83% NH_4Cl (10 min, 4°C) and washed. The leukocyte pellet was resuspended in PBS supplemented with 0.2% heat-inactivated FBS. Viability of cells isolated in this manner was >95% when assessed by trypan blue or propidium iodide exclusion.

Membrane Potential Assay

Alterations in membrane potential were determined by measuring changes in the intracellular concentrations of DiOC5(3), which partitions between cells and aqueous media as a function of transmembrane potential. Cellular fluorescence intensity varies as a function of membrane potential, decreasing as the membrane depolarizes. PMN aliquots (10^6 cells/ml) were resuspended in glucose-supplemented (5 mg/ml) HBSS and incubated with 10^{-8} *M* DiOC5(3) for 10 min at 37°C. Cells aliquots were stimulated with PMA (100 ng/ml) or XO (0.5 U/ml) for 10 min at 37°C. Changes in resting membrane potential (t = 0 min) were analyzed by flow cytometry using a FACS analyzer interfaced to a Consort 30 computer system. Green fluorescence was monitored between 515 and 545 nm after excitation by a mercury arc lamp equipped with a 485/22 nm excitation filter. PMNs were distinguished from other cellular types based on coulter volume and right-angle light scatter properties. Quantitation of membrane depolarization was determined by the following formula:

(fluorescence at 0 min − fluorescence at 10 min)/fluorescence at 0 min

Measurement of Intracellular H_2O_2 Production

H_2O_2 production was measured as described previously by Bass et al. (10). Cells (10^6/ml) were incubated for 10 min at 37°C with 5 μ*M* DCFH-DA in Ca^{2+}/Mg^{2+}-free PBS supplemented with 0.2% FBS. DCFH-DA diffused through cellular membranes and was hydrolyzed by cellular esterases to nonfluorescent 2′,7′-DCFH, which is intracellularly trapped. Cells were then stimulated with PMA, uricase, or XO and analyzed. Cells capable of producing intracellular H_2O_2 oxidize DCFH to the fluorescent analogue DCF. PMNs were distinguished from other cellular types on the basis of coulter volume(s) and right-angle light scatter properties. DCF levels were measured as described for membrane potential, and the percent change in H_2O_2 production was determined by the following formula:

$$100 \times [(\text{mean fluorescence intensity (FL) experimental} - \text{FL control})/\text{FL control}]$$

Statistical Analysis

All data are presented as the mean ± SEM. Statistical differences were determined using Student's *t* test, and any p values <0.05 were considered statistically significant.

RESULTS

PMA alters the membrane potential of isolated, normal peripheral canine neutrophils. Assays of individual samples were run in triplicate. Flow cytometric analyses of membrane potential changes demonstrated that 89–96% of the cellular population (determined by right-angle light scatter to be granulocytes) was depolarized at 10 min between 32 and 48% (change in cellular fluorescence × 100) with

an average change of 41% in comparison with 37°C controls (Table 1). Quantitatively, although fewer cells were stimulated by XO (range 54–86%, mean 70%) than by PMA, responding cells exhibited a substantially increased depolarization (range 76–90%, mean 70%).

Figure 1 depicts cytosolic H_2O_2 that was stimulated by XO at final concentrations of 0.025–0.5 U/ml when examined at 15 min. Figure 2 demonstrates cellular H_2O_2 production of PMNs examined 10–35 min after stimulation with 0.5 U XO/ml. H_2O_2 production was apparent for 15 min, after which the responses begin to decrease.

DISCUSSION

The in vitro biological effects of XO on canine PMNs include significant membrane and metabolic effects. In comparison with membrane depolarization characteristics demonstrated by PMA, XO involved fewer PMNs but at significantly enhanced response levels. In addition to the membrane effects, XO also enhanced PMN H_2O_2 production three times that of PMA maxima.

Alterations in cellular transmembrane potentials have been suggested as being among the earliest events following appropriate levels of stimulus-response coupling (11). DiOC5(3) and other closely related lipophilic cations are able to assess the electrophysiological state of cellular membranes by differences in potential-dependent fluorescent partitioning coefficients. PMA stimulates a loss of cellular fluorescence representative of electrophysiological membrane depolarization and has been used by investigators as a reference standard (12). In vitro, XO induced greater effects on PMN membranes than did PMA and although reacting with fewer PMNs was able to depolarize them to a greater extent.

Different lots of XO have been examined and, although active (compared with controls), have demonstrated significant variability. Older lots of XO demonstrated the highest activities. Recent lots of XO, although significantly active, were unable to stimulate to PMA maxima. The liquid vehicle (2.3 *M* ammonium

TABLE 1. *Alterations in canine neutrophil membrane potentials following stimulation by phorbol myristate acetate (PMA) or xanthine oxidase (XO)*

	PMA[a]		XO[b]	
Subject	Response	Percent early responders	Response	Percent early responders
1	0.32	89	0.81	72
2	0.38	90	0.81	79
3	0.45	96	0.85	86
4	0.46	95	0.76	62
5	0.36	94	0.81	63
6	0.48	89	0.84	54
7			0.90	61
8			0.87	84
Mean ± SEM	0.41 ± 0.03	92 ± 1	0.83 ± 0.02	70 ± 4

[a] PMA, final concentration 100 ng/ml.
[b] XO, final concentration 0.5 U/ml.

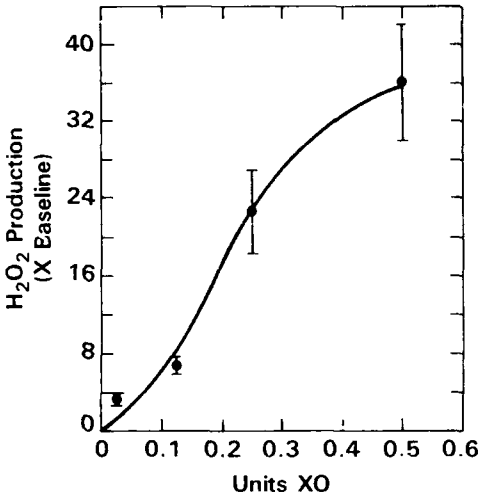

FIG. 1. Canine PMN H_2O_2 production (×baseline) following 15 min stimulation with xanthine oxidase (XO) between 0.0025 and 0.5 U/ml. Data points represent the mean fluorescense intensity (±SEM) of a minimum of 10,000 events gathered from 12 to 21 animals.

sulphate) had no effect on PMN cellular membranes or metabolism (results not shown). Uricase, a common enzyme contaminant of XO preparations, was determined to have small, significant, direct effects and little or no priming ability (results not shown).

The reasons for the lot-to-lot manufacturer variability will require further investigation. Despite the reagent variability, XO remains an interesting biological moiety based on these results and others suggesting substantial XO increases in mice following their inoculation with Ehrlich carcinoma cells, bacteria, or protozoa (13). The mechanisms by which XO participates within inflammatory pro-

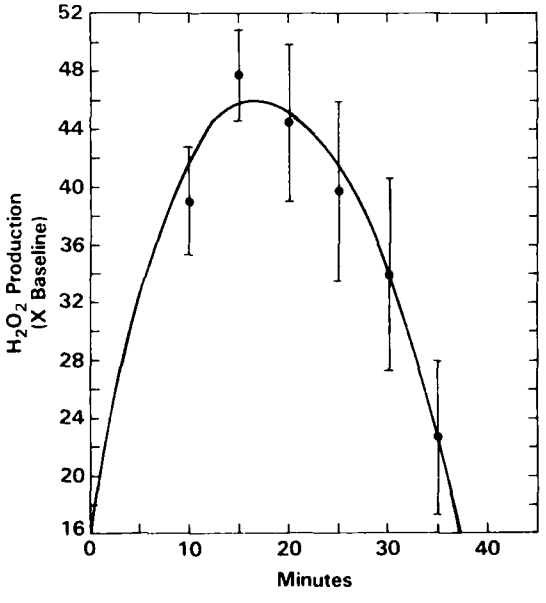

FIG. 2. Canine PMN H_2O_2 production (×baseline) between 10 and 35 min following stimulation with 0.5 U XO/ml. final concentration. Data points represent the mean fluorescence intensity (±SEM) of a minimum of 10,000 events from 10 to 12 animals.

cesses deserve further examination, since inappropriate in vivo XO levels could generate toxic oxygen radical forms and destruction of healthy tissue.

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