0031-3998/93/3403-0303$03.00/0
PEDIATRIC RESEARCH
Vol. 34, No. 3, 1993
Printed in U.S.A.
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Physiologic Levels of Uric Acid Inhibit Xanthine Oxidase in Human Plasma

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ABSTRACT. Xanthine oxidase, a key source of reactive oxygen species, and purine substrates are detected in the circulation after ischemia-reperfusion. High levels of uric acid, produced by a xanthine oxidase–catalyzed reaction, are found in human plasma. We studied whether uric acid could alter xanthine oxidase activity in plasma obtained from eight adults and eight neonates. Known amounts of uric acid were added to xanthine and xanthine oxidase–supplemented buffer and plasma, and the production of uric acid and superoxide was determined. Uric acid, 150 and 300 μM, decreased the oxidation of xanthine to uric acid in adult plasma by 37.5 ± 5.6 and 48.9 ± 6.1% and formation of superoxide by 23.2 ± 1.9 and 32.0 ± 2.3%, respectively, compared with plasma without uric acid. In newborn plasma, a similar pattern and extent of inhibition was observed. Superoxide formation, however, was inhibited to a greater extent than in adult plasma. Endogenous xanthine oxidase was detected in newborn plasma in nine additional neonates using HPLC. These results indicate that uric acid is an effective inhibitor of the formation of superoxide and hydrogen peroxide by xanthine oxidase at the levels found in human plasma. Plasma uric acid may play an important role in attenuating the oxidant-mediated tissue damage caused by xanthine oxidase released into the circulation during ischemia-reperfusion. (*Pediatr Res* 34: 303-307, 1993)

Abbreviations

H_2O_2, hydrogen peroxide
O_2^-, superoxide
SOD, superoxide dismutase
UA, uric acid
XDH, xanthine dehydrogenase
XO, xanthine oxidase

UA, because of its capacity to act as an antioxidant and free-radical scavenger, plays an important role in biologic fluids (1-3). Plasma levels of UA increase with advancing age, with the lowest levels of UA seen in the childhood years (4). UA levels are, however, elevated in premature infants, varying inversely with gestational age (4). XO, the enzyme involved in the production of UA, is a source of production of the oxidants, O_2^-, and H_2O_2. These oxidants may be involved in the pathogenesis of the tissue injury associated with many disease states, including reperfusion of ischemic tissues (5). XO activity has been detected in tissues at a gestational age (6, 7) that may precede the maturation of enzymatic antioxidant defenses to adult levels in tissues (8), implicating the need for nonenzymatic antioxidant defenses. Xanthine oxidoreductase (EC 1.2.3.2), existing in healthy cells as the NAD$^+$-reducing XDH, is converted to oxygen radical-producing XO during ischemia (5). XDH and XO are released in the circulation in many animal models (9, 10), in hemorrhagic shock (11), after ischemia (12), and after tourniquet injury to a human limb (13). Cellular ATP is catabolized during ischemia or oxygen deficiency, resulting in elevated hypoxanthine levels in plasma during tissue hypoxia (14). Upon reperfusion (reoxygenation), XO can react with purine substrates (hypoxanthine or xanthine) and molecular oxygen to produce UA and, in one- and two-electron reduction steps (univalent and divalent flux), O_2^- and H_2O_2, respectively (15-17).

The absence of enzymes responsible for further metabolism of UA in humans results in plasma UA concentrations as high as 500 μM (4). We asked whether UA could have a biologic role, other than that of an antioxidant, as an inhibitor of circulating XO activity. Although UA has been known to inhibit XO in alkaline chemical solutions and at high concentrations (≥670 μM) (18, 19), little is known about its behavior in biologic fluids with normal pH. We hypothesized that the levels of UA found in plasma would inhibit XO activity in plasma. This study investigated the effect of UA in plasma on the oxidation of xanthine to UA and the amount of univalent-divalent flux. We also compared the effect of UA inhibition in newborn plasma with that in adults to determine whether UA was an effective inhibitor of XO in newborn plasma.

MATERIALS AND METHODS

Bovine milk XO was obtained from Calbiochem (La Jolla, CA). Stock solutions were made daily by centrifuging 25 μL of XO suspended in 2 M $(NH_4)_2SO_4$ for 2 min in a microcentrifuge at 5000 × *g*. The supernatant was discarded and the sedimented crystals resuspended in 50 mM potassium phosphate buffer, 0.1 mM EDTA, pH 7.4. Bovine erythrocyte Cu-Zn SOD was obtained from Grünenthal (Aachen, Germany). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) except acetonitrile, which was obtained from Baxter Healthcare Corp. (McGaw Park, IL). Blood was collected from eight healthy adult volunteers (20–40 y old) and eight healthy full-term newborn babies at delivery. The sites of blood collection were the antecubital vein in adults and the umbilical cord artery and vein in neonates. Blood was pipetted into heparinized tubes, placed on ice, and centrifuged immediately. The plasma was chromato-

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Received July 20, 1992; accepted April 23, 1993.

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Supported by the National Institutes of Health Grant R29-DK-38681 and P01-HL-48676, and Grant AHA-870029 from the Alabama Affiliate of the American Heart Association.

graphed on a Sephadex G-25 column to remove endogenous purines and low molecular weight inhibitors (postcolumn plasma). Plasma protein levels were measured before and after the column procedure by the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL). The concentration of plasma was adjusted so that the final concentration of plasma protein in the cuvette was always 15 g/L (±0.1 g/L). This was done to control for the variability of the dilution from the chromatography of different plasma samples. Measurements of oxidation of xanthine to UA and O_2^- production by XO were made in the presence or absence of plasma on a Gilford spectrophotometer (Ciba Corning Diagnostics, Oberlin, OH). A mean of three measurements was obtained for every assay.

XO activity measurement. The activity of XO was measured daily by monitoring the absorbance change at 292 nm (for UA production) in the presence of 50 μM xanthine and 50 mM potassium phosphate buffer with 0.1 mM EDTA (pH 7.4) (20). The amount of XO was adjusted to a final activity of 5 mU/mL (± 0.02 mU/mL).

In the measurements involving plasma, postcolumn plasma was mixed with 50 mM potassium phosphate buffer (pH 7.4) with 0.1 mM EDTA, 5 mU/mL XO, 50 μM xanthine, and 0–300 μM UA at 25°C. The amount of UA formed from xanthine was quantified by using a spectrophotometer and monitoring the absorbance at 308.5 nm (21). This wavelength was chosen because plasma proteins cause less interference with the detection of UA at this wavelength than at 292 nm. The extinction coefficient of UA at 308.5 nm was 3085 $M^{-1} \cdot cm^{-1}$ at 25°C and pH 7.4. Because of limitations in the amount of blood obtained from newborn samples, we were able to study only three concentrations of UA in all subjects. In the umbilical artery group, six subjects were studied because we were unable to obtain blood from two of the subjects. With UA concentrations of 500 μM, absorbance values greater than 2 were obtained in plasma that exceeded the linearity of the spectrophotometer. This resulted in unacceptable variations in the rate of change of absorbance. In buffer, UA concentrations of 500 μM did not present any problems. An intermediate concentration of 200 μM was studied in some plasma samples to confirm the pattern of inhibition and is not presented in the figures.

O_2^- determination. O_2^- was determined from the SOD-inhibitable reduction of ferricytochrome *c* at 550 nm, in the presence and absence of postcolumn plasma. Postcolumn plasma was mixed with 50 mM potassium phosphate buffer (pH 7.4) with 0.1 mM EDTA, 50 μM xanthine, 5 mU/mL XO, 100 μM ferricytochrome *c*, and 0–300 μM UA, at 25°C. The reduction of ferricytochrome *c* from non-O_2^- sources was determined by the addition of 1 μM SOD to the above reaction. Thus, the amount of O_2^- formed from the reaction of XO with xanthine was obtained from the difference of the two values, with and without SOD.

Endogenous XO activity in newborn plasma. Nine additional healthy full-term neonates were studied for the presence of circulating XO. We were unable to detect endogenous XO activity consistently in newborn plasma with use of a spectrophotometer because its lower limit is 0.5 mU/mL plasma. Thus, we developed an HPLC method with electrochemical detection that could detect one-thousandth the activity detected by the spectrophotometric method. Plasma was obtained from both umbilical artery and vein. Total XDH + XO and XO activity was determined by the addition of xanthine (75 μM) to postcolumn plasma, with and without the addition of 500 μM NAD^+, respectively. The plasma was incubated at 37°C for 60 min and the reaction stopped by addition of cold 99.9% acetonitrile. The precipitate was removed by centrifugation (6000 × *g*, 20 min) and the supernatant evaporated by a Speed Vac evaporator (Savant Instruments, Farmingdale, NY). The residue from evaporation was resuspended to the original plasma volume with 50 mM sodium acetate buffer, pH 4.75. Fifty μL was then injected onto a 5-μm C-18 column (15 × 0.46 cm) (Perkin-Elmer, Norwalk, CT) with a mobile phase of 50 mM sodium acetate buffer, pH 4.75, and a flow rate of 1.0 mL/min. The amount of UA formed from xanthine was quantified using an electrochemical detector (Coulochem 5100A, ESA, Bedford, MA). The UA in plasma was confirmed by using the retention time of UA standard in 50 mM sodium acetate buffer, pH 4.75. The quantities of UA were determined by comparison of the peak areas to those of standards. A unit of enzyme activity was defined as 1 μmol of UA formed per minute at 37°C and pH 7.4.

Statistical analysis. Analysis was done by paired *t* test for comparison of buffer and plasma and by two-sample *t* test for comparison of two types of plasma. Analysis of the effect of UA on xanthine oxidation and O_2^- formation in both buffer and plasma was conducted with repeated measures analysis of variance (22). For studies in buffer that included 0–500 μM of UA, a nonlinear regression procedure using a least-square estimate of the parameters of a nonlinear model was used (22). The model, $y = a(e^{-bx}) + c$, was fitted by a modified Gauss-Newton method, where y = xanthine oxidation or O_2^- formation, x = UA concentration, and a, b, and c are partial derivatives of the model. For the analysis of the similarity of the trend between xanthine oxidation and O_2^- formation, a nonlinear model was fitted including the adjusted values of xanthine oxidation and O_2^- formation. From the residual sum of squares of the three models, an *F* statistic was calculated. An alpha error of less than 0.05 was considered significant. All results are expressed as mean ± SD.

RESULTS

Inhibition in adult plasma. UA inhibited XO activity in plasma at 150 and 300 μM by 37.5 ± 5.6 and 48.9 ± 6.1%, respectively, compared with plasma without UA. In buffer, a similar pattern was found: XO activity at the same UA concentrations was 32.4 ± 2.5 and 41.7 ± 3.0%, respectively, less than the activity without UA (Fig. 1). The decrease in O_2^- production by UA was 23.2 ± 1.9 and 32.0 ± 2.3% at 150 and 300 μM, respectively, in plasma. In buffer, the decrease was 21.0 ± 3.2 and 30.5 ± 2.7%, respectively, at the same concentrations (Fig. 2).

Inhibition in newborn plasma. UA decreased the xanthine

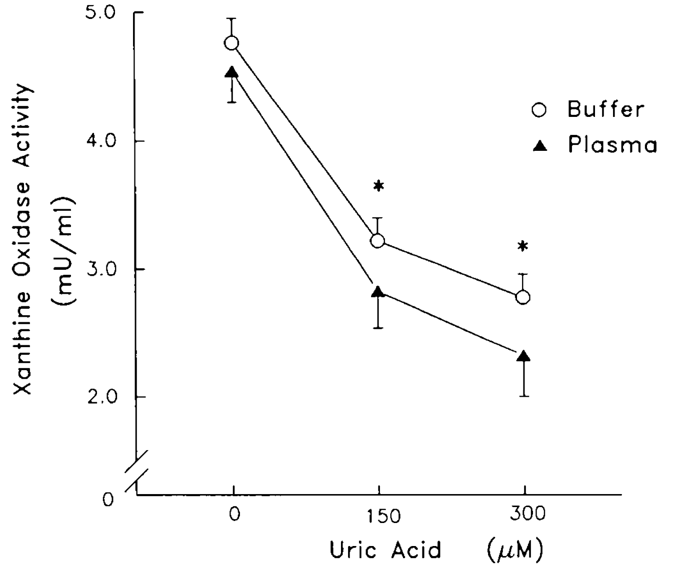

Fig. 1. Comparison of XO activity (±SD), determined by xanthine to UA turnover, in adult plasma ($n = 8$) and buffer (paired with plasma, $n = 8$). Each sample was assayed in triplicate. UA caused a decrease in XO activity with increasing concentration of UA in both plasma (analysis of variance, $p < 0.0001$) and buffer ($p < 0.0001$). The pattern of inhibition is not different between plasma and buffer groups (repeated measures analysis of variance, NS). At 150 and 300 μM UA concentrations, plasma activity is less than that in buffer (*, *t* test, $p < 0.05$).

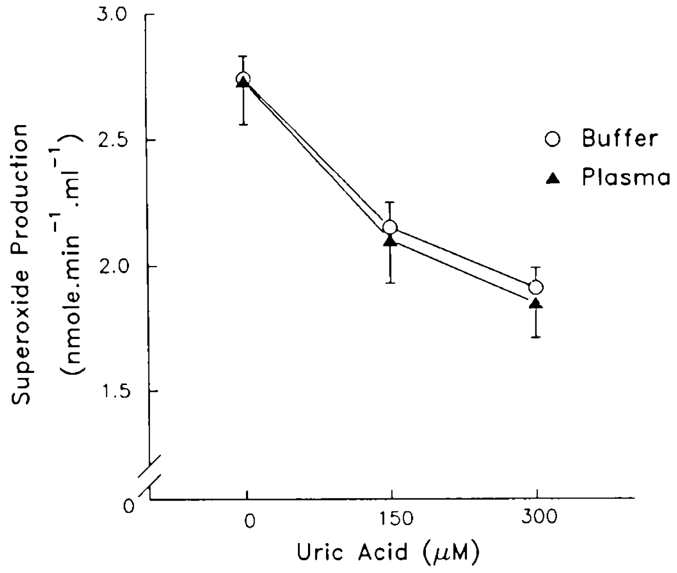

Fig. 2. Comparison of O_2^- formation by XO (±SD) in adult plasma and buffer (see Fig. 1 for details). UA caused a decrease in formation of O_2^- in both plasma ($p < 0.0001$) and buffer ($p < 0.0001$). There was no difference in the pattern of inhibition or at each UA concentration between the plasma and buffer groups.

oxidation (XO activity) and the production of O_2^- by XO in both newborn plasma and buffer (Fig. 3). UA, 200 μM, in both plasma and buffer confirmed the pattern of inhibition (data not shown). Only umbilical vein plasma results are shown. Umbilical artery plasma studies ($n = 6$) showed no significant difference from the umbilical vein plasma studies.

Comparison of trends of inhibition in buffer. In buffer, xanthine oxidation and O_2^- formation at 500 μM UA was also studied in addition to 0, 150, and 300 μM. A nonlinear trend was noted in both the xanthine oxidation and O_2^- formation, as expected. Using a nonlinear regression procedure, the decrease of xanthine oxidation by UA was not significantly different from the decrease of O_2^- formation by UA. We were unable to evaluate the effect of 500 μM UA in plasma because of unacceptable variations in rate of change of absorbance on the spectrophotometer at these high absorbance values (absorbance values ≥2).

Comparison of adult and newborn plasma. Although the pattern of XO inhibition by different concentrations of UA was not different between plasma and buffer, there were differences noted in XO activity at individual UA concentrations. Less XO activity was found in adult plasma than in buffer at 150 and 300 μM, in contrast to the findings in newborn plasma of increased XO activity compared with buffer (Figs. 2 and 3*A*). Less O_2^- production occurred at all concentrations of newborn plasma compared with buffer (Fig. 3*B*). When expressed as a percentage of the buffer control, XO activity was significantly higher in newborn plasma than in adult plasma (Fig. 4). O_2^- produced by XO in newborn plasma was significantly lower than in adult plasma.

Univalent flux describes single electron transfers from the enzyme XO to O_2 to form O_2^- compared with divalent flux, which describes the transfer of two electrons to O_2 to form H_2O_2 (15–17). UA increased the univalent flux in adult plasma in a dose-dependent fashion but did not change the univalent flux significantly in newborn plasma (Table 1).

Endogenous XO in newborn plasma. To explain the apparent discrepancy between adult and newborn plasma, we tested for the presence of endogenous XO in newborn plasma. We found elevation of total (XDH + XO) and XO activity in both umbilical artery and umbilical vein plasma (Table 2).

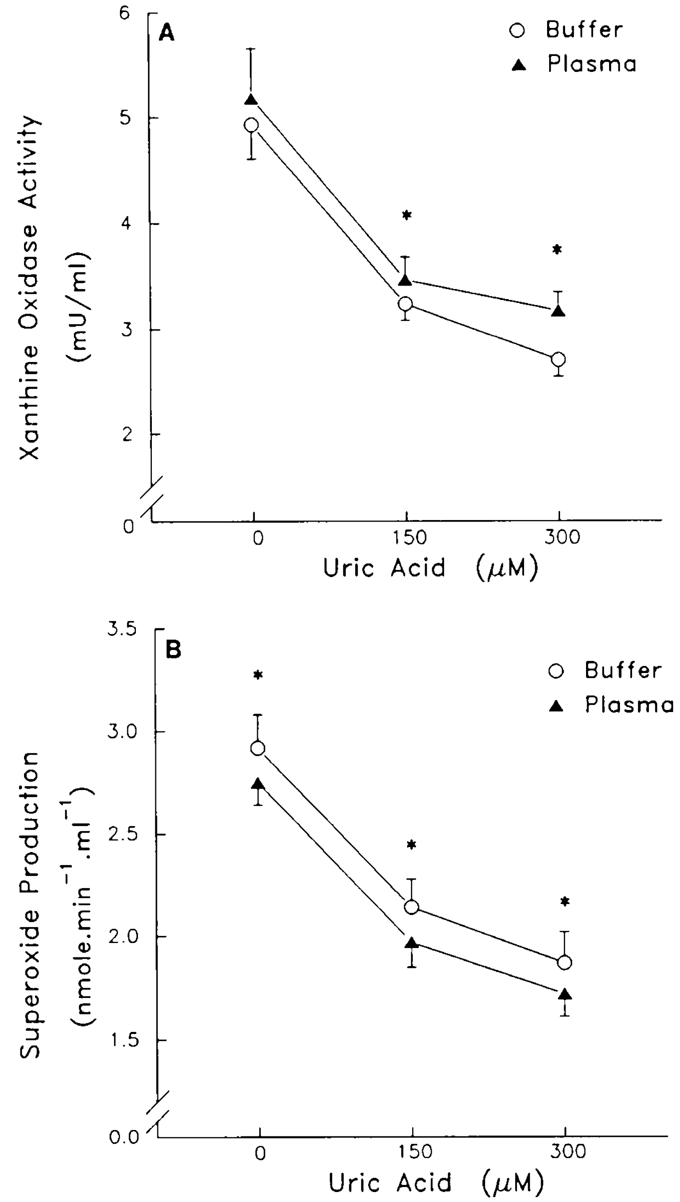

Fig. 3. Comparison of XO activity, determined by xanthine oxidation (*A*), and O_2^- production by XO (*B*) in newborn plasma ($n = 8$) and buffer (paired with newborn plasma, $n = 8$). Each sample was assayed in triplicate. UA caused a decrease in xanthine oxidation and production of O_2^- in both newborn plasma and buffer ($p < 0.0001$ in all cases). There was no difference between the plasma and buffer groups in *A* or *B*. Note differences between plasma and buffer at each UA concentration (*, $p < 0.05$).

DISCUSSION

The present study indicates that the high levels of UA (150–500 μM) normally found in human plasma constitute an important defense mechanism by which the production of reactive oxygen metabolites by circulating XO is attenuated. Circulating XO has been demonstrated in the circulation after ischemia-reperfusion in many animal models (9–11) and in humans after upper limb ischemia (12, 13) and liver transplantation (23). XO has also been detected in significant quantities in the systemic circulation secondarily to diverse pathologic processes including adult respiratory distress syndrome and thermal injury (24, 25). We demonstrated that UA inhibits the oxidation of xanthine to UA and decreases the formation of O_2^- by XO.

The levels of hypoxanthine, another substrate of XO, increases to ~25 μM in hypoxia in the newborn (14). In our study, excess substrate (xanthine) concentration was selected to ensure that XO activity was at V_{max}. Substrate excess in plasma mimics the worst clinical situation where the release of substrate is far in

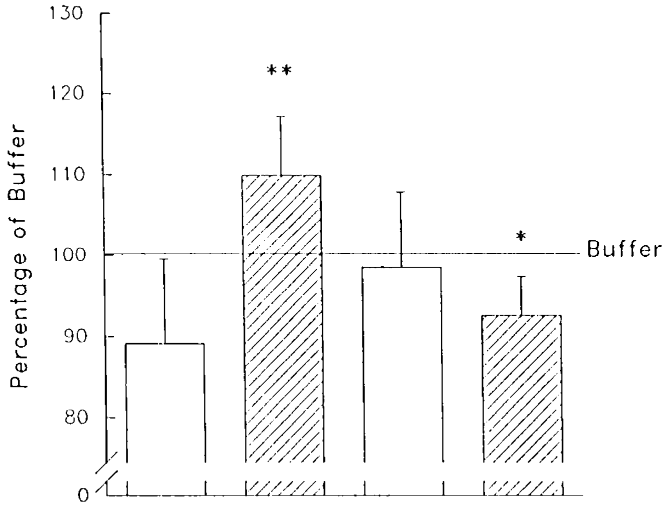

Fig. 4. Comparison between adult and newborn plasma of XO activity (**, $p < 0.0001$) and O_2^- formation (*, $p < 0.01$), expressed as percentage of corresponding buffer controls.

Table 1. *Univalent flux, expressed as percentage of total electron flux, in adult and newborn plasma with and without uric acid**

Uric acid concentration (μM)	Adult plasma (%)	Newborn plasma (%)
0	30.1 ± 2.5	25.1 ± 1.0
150	37.4 ± 5.5	26.9 ± 2.3
300	40.8 ± 7.4	26.2 ± 2.4

Uric acid increased univalent flux in adult plasma in a dose-dependent fashion ($p < 0.004$) but did not change univalent flux significantly in newborn plasma.

Table 2. *Endogenous XDH + XO and XO activity in plasma obtained at delivery from umbilical cord artery and vein*

	Umbilical artery	Umbilical vein
Total (XDH + XO) (μU/mL)	8.9 ± 2.5	9.6 ± 2.1
XO (μU/mL)	2.4 ± 0.5	2.5 ± 0.3

excess of the Km (substrate concentration at half-maximal velocity) of XO (4–6 μm). Lower substrate concentrations, on the other hand, result in submaximal velocities for XO activity (26). This, along with high UA concentrations, produces a greater net inhibition of XO activity. Thus, UA may play a greater protective role in normal neonates and in conditions of mild hypoxia.

UA is an important antioxidant and free radical scavenger in biologic systems (2, 3). Antioxidants may play an important role in the slowing of the aging process (1, 27, 28). Elevations in plasma UA in man are found in adulthood and in premature neonates (4). In premature babies, plasma UA levels have an inverse relationship with gestational age. The more premature the baby, the more elevated is the UA level in plasma (4). After birth, UA levels drop, with the lowest concentrations being found in the school age years, and then increase in the teen-age years through adulthood. Thus, it is interesting to note that elevations in plasma UA occur at the ends of the life span in man. The increases coincide with periods of oxidant stress (in adulthood) or with periods of relative vulnerability to oxidants (premature gestation).

The levels of many antioxidant enzymes, such as SOD, catalase, and glutathione peroxidase, increase only close to the time of delivery and reach adult levels only after birth (8, 29). However, the oxidant-generating enzyme, XO, is detected in liver and intestine as early as 20 wk of gestation (6, 7). The capacity for the generation of reactive oxygen species may be present at an earlier stage of development than the appearance of adequate levels of antioxidant enzymes (29). Thus, UA may play an important protective role, not only as an antioxidant, but as an inhibitor of XO in premature babies. Further work is in progress to elucidate the relationship between the oxidant-generating systems and antioxidant levels during development in humans.

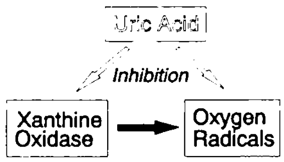

Fig. 5. UA is both an antioxidant as well as an inhibitor of XO in plasma.

H_2O_2 and O_2^- are two of the reduced species formed by the reaction of XO with xanthine or hypoxanthine. The amount of reduction in O_2^- formation (univalent reduction) is less than the inhibition of xanthine oxidation to UA by XO. This suggests that the extent of decrease in H_2O_2 formation by XO (divalent reduction) is greater than the extent of decrease in univalent reduction. Therefore, less H_2O_2 is formed than O_2^- in the presence of UA.

XO levels were consistently higher in newborn plasma and lower in adult plasma than measured in buffer. This is the first study that has demonstrated the presence of endogenous XO in normal newborn plasma, but the levels detected were too low to explain the difference between adult and newborn plasma. No significant difference in XO levels was found at zero UA concentration. Eight percent of plasma IgG from healthy adult humans has been found to be immunoreactive with XO and to partially inhibit enzyme activity (30). It is not known whether anti-XO IgG levels differ in newborn plasma. The amount of univalent flux was also lower in newborn plasma than in adult plasma. This would imply that the ratio of H_2O_2 to O_2^- produced by XO is higher in newborn plasma than in adult plasma. Increasing UA concentrations seemed to increase the univalent flux in adult plasma but not in newborn plasma. Further investigations are needed to elucidate the mechanism of these differences.

The role of UA can be summarized as both a direct scavenger of oxygen radicals as well as an inhibitor of the formation of O_2^- and H_2O_2 by XO (Fig. 5) at the levels found in human plasma.

Acknowledgment. This manuscript is dedicated to the memory of Roy Alan Evans, who provided invaluable assistance in this research.

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