Plasma Xanthine Oxidase Activity and Lipid Hydroperoxide Levels in Preterm Infants

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ABSTRACT

Ischemia-reperfusion injury may affect morbidity and mortality in preterm and asphyxiated term infants. Reoxygenation of hypoxic tissues leads to the formation of free oxygen radicals by xanthine oxidase that may induce lipid peroxidation, enzyme inhibition, and DNA strand breakage. We measured arterial cord blood samples from 36 healthy term infants for baseline values and arterial blood sampled at 1 and 4 h after birth from 45 preterm infants admitted for intensive care for serial estimates of plasma xanthine oxidase activity and lipid hydroperoxide levels. Mean $\pm$ SEM plasma xanthine oxidase activity in cord blood of term infants was 2.3 ± 0.4 mU/mL and lipid hydroperoxide levels were 2.6 ± 0.3 nmol/mL. Eighteen of the 45 preterm infants met the criteria defining poor outcome (poor outcome group) and had lower umbilical arterial pH and base excess than the 27 preterm infants in the control group. Mean plasma xanthine oxidase activity in-

creased from 2.7 ± 0.4 at 1 h to 4.7 ± 0.6 mU/mL at 4 h of age ($p < 0.001$) in the poor outcome group and decreased from 2.1 ± 0.3 to 1.1 ± 0.2 mU/mL ($p = 0.004$) in the control group. Lipid hydroperoxide levels in the poor outcome group increased from 2.8 ± 0.6 nmol/mL at 1 h to 4.3 ± 0.6 nmol/mL at 4 h of age ($p < 0.001$) and decreased from 2.1 ± 0.6 to 1.6 ± 0.2 nmol/mL ($p = 0.008$) in the control group. At 4 h of age, xanthine oxidase activity and lipid hydroperoxide levels were significantly higher in the poor outcome group than in the controls ($p < 0.001$). We conclude that serial measurements of plasma xanthine oxidase activity and lipid hydroperoxide levels may identify critically ill preterm infants destined for major neonatal morbidity and mortality and may be clinically useful as an indicator of severity of acute perinatal hypoxia-ischemia. (*Pediatr Res* 36: 283–287, 1994)

Free oxygen radicals play a major role in ischemia-reperfusion or hypoxia-reoxygenation injury (1–6). During ischemia or hypoxia, the continuing use of ATP is not balanced by production of ATP, leading to degradation of energy-rich phosphates from ATP to AMP and further to adenosine. Adenosine is able to leave the cell and will be further degraded to inosine and hypoxanthine. Normally, hypoxanthine is metabolized to xanthine and xanthine to urate by the enzyme xanthine dehydrogenase with the use of NAD^+ as cosubstrate. Ischemia induces the conversion of xanthine dehydrogenase to xanthine oxidase by proteolytic modification and oxidation of sulfhydryl groups (7). Xanthine oxidase is an important source of free oxygen radicals, especially superoxide anions (8), because it is able to use molecular oxygen instead of NAD^+ as electron acceptor in the two-step conversion of hypoxanthine to uric acid. This reaction mechanism has

been confirmed in both animal and human studies (9–12) and is probably the primary source of reactive oxygen species after reperfusion of ischemic tissues (13). Release of xanthine oxidase in sick newborns was first hypothesized by Saugstad *et al.* in 1984 (14). Recent studies have shown a high xanthine oxidase activity in the fetal liver and intestine throughout gestation that increases in the liver and decreases in the intestine with advancing gestation (15) and have demonstrated the presence of endogenous xanthine oxidase in normal newborn plasma (16). Hypoxanthine, the main substrate of xanthine oxidase, has been proposed as an important marker for perinatal asphyxia (9, 12, 17, 18). Hypoxanthine levels were found to be increased in the vitreous humor of preterm infants who died of severe asphyxia (19) and in cord plasma of infants who experienced perinatal asphyxia (12, 17), suggesting that reactive oxygen species are instrumental in the compromised outcome of newborn infants after an asphyxial insult.

The deleterious effects of free oxygen radicals are secondary to lipid peroxidation, enzyme inhibition, and DNA strand breakage (4). Of these processes, the measurement of products of lipid peroxidation has been widely

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used in biologic studies to identify the occurrence of oxidant injury (20).

Growing evidence suggests that free oxygen radicals play a significant role in the development of morbidity and mortality in preterm infants and that major conditions such as bronchopulmonary dysplasia, pulmonary hemorrhage, retinopathy of prematurity, and necrotizing enterocolitis may in part be attributable to oxidant injury (21). To substantiate this claim, we tested the hypothesis that preterm infants with a severely compromised early neonatal outcome have experienced oxidant injury. We assumed increased xanthine oxidase activity to be the primary source of free oxygen radicals and the presence of plasma lipid hydroperoxides to reflect tissue damage from oxidant injury.

METHODS

Study population. Arterial cord blood samples were collected from 36 healthy term infants and arterial blood samples at 1 and 4 h after birth from 45 preterm infants with a gestational age less than 32 wk admitted to our neonatal intensive care unit. The records of the latter infants were periodically reviewed to identify "poor outcome," defined as the occurrence of any of the following: early neonatal death, pulmonary hemorrhage within the first week after birth, presence of neuropathology (intra/periventricular hemorrhage grade III or IV, seizures) within the first week after birth, or necrotizing enterocolitis (positive abdominal x-ray or bowel perforation surgery) within the first 2 wk after birth. The infants with 1- and 4-h measurements were divided into two groups based on their outcome, *i.e.* those who met the poor outcome criteria (poor outcome group) and those who did not (control group).

This study was approved by the Institutional Review Board and written parental informed consent was obtained before the collection of blood samples.

Methods. All reagents were obtained from Sigma Laboratories (St. Louis, MO), except for perchloric acid, which was obtained from Aldrich Laboratories (Milwaukee, WI), and the LPO determiner kit, which was obtained from Kamiya Biomedical (Thousand Oaks, CA).

One mL of arterial blood was collected in ice-cold EDTA tubes and mixed with 100 μ L of a solution containing 10 mM EDTA, 10 mM dithioerythritol, and 10 mM phenylmethyl-sulfonyl fluoride in phosphate buffer (pH 7.35) to prevent inactivation or conversion of xanthine dehydrogenase to xanthine oxidase *in vitro*. The samples were transported on ice and spun at 1500 $\times g$ for 15 min at 4°C to separate the plasma from the red blood cells. The plasma was divided into two parts: one part was frozen at -20°C for lipid hydroperoxide assay within 1 wk, and one part was immediately processed for xanthine oxidase activity.

Xanthine oxidase activity. The plasma samples were passed over a 10-mL Sephadex G-25 column of 4°C, preequilibrated with phosphate buffer with DTT, phenyl-

methylsulfonyl fluoride, and EDTA to remove endogenous substrates and low molecular weight inhibitors before measuring xanthine oxidase activity (22). The cord blood samples were assayed for xanthine oxidase activity using both spectrophotometric monitoring of the conversion of hypoxanthine (100 μ M in phosphate buffer) to urate at 295 nm (23) (1 unit of xanthine oxidase forms 1 μ mol of urate from hypoxanthine/min at 25°C, pH 7.9) and the oxidation of the chromogen ABTS [2, 2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonate)] by hydrogen peroxide generated in the two-step oxidation of hypoxanthine to urate with oxygen as electron acceptor at 410 nm (24). Baseline values were obtained for each sample by incubating samples with substrates and allopurinol (50 μ M). The two methods had an excellent correlation ($r = 0.99$, $p < 0.001$), and the ABTS technique was used exclusively for measurement of xanthine oxidase activity in the 1- and 4-h arterial samples collected from the 45 preterm infants. Specific xanthine oxidase activity was expressed as mU/mL plasma.

Lipid hydroperoxide assay. Lipid hydroperoxide levels were measured using the observation (25) that Hb catalyzes the reaction of lipid hydroperoxides with the methylene blue derivative MCDP (10-N-methylcarbamoyl-3,7-dimethylamino-10-H-phenothiazine), forming an equimolar concentration of methylene blue with maximum absorbance at 675 nm (LPO determiner kit, Kamiya Biomedical). Cumene hydroperoxide was used as external standard in the assay. Lipid hydroperoxide levels were expressed as nmol/mL plasma.

Statistics. Results are presented as mean $\pm$ SEM. The means of two groups were compared with the *t* test, and paired *t* tests were used to compare the means of repeated measures. The association between birth weight/gestational age and both xanthine oxidase activity and lipid hydroperoxide levels was determined by multiple regression analysis. A *p* value less than 0.05 indicated statistical significance.

RESULTS

Table 1 summarizes the characteristics of the 36 healthy term newborn infants studied for arterial cord plasma xanthine oxidase activity and lipid hydroperoxide levels. Their mean xanthine oxidase activity was $2.3 \pm$

Table 1. Characteristics of the 36 healthy term newborn infants studied for cord plasma xanthine oxidase activity and lipid hydroperoxide levels

	Mean $\pm$ SEM	Range
Birth weight (g)	3580 $\pm$ 109	2290-4500
Gestational age (wk)	39.2 $\pm$ 0.4	37-42
Vaginal/cesarean section delivery*	23/13	
Males/females*	16/20	
5-min Apgar score†	9	9
Arterial umbilical pH	7.28 $\pm$ 0.01	7.22-7.37

* Absolute values.

† Median values.

Table 2. Characteristics of the 45 preterm infants admitted for intense care in whom 1- and 4-h plasma xanthine oxidase and lipid hydroperoxide levels were measured

	Poor outcome group (n = 18)	Controls (n = 27)	p
Birth weight (g)	930 ± 42	1054 ± 32	0.02
Gestational age (wk)	27.3 ± 0.4	28.7 ± 0.3	0.009
5 min Apgar score*	7	9	0.003
Art umbilical pH	7.22 ± 0.02	7.31 ± 0.01	<0.001
Base excess	-8.8 ± 0.9	-2.8 ± 0.4	<0.001
RDS† and mechanical ventilation > 48 h	15	22	NS
Early neonatal death‡	10	0	<0.001
Pulmonary hemorrhage <1 wk after birth	2	0	0.07
Neuropathology <1 wk after birth	10	0	<0.001
Necrotizing enterocolitis <2 wk after birth	6	0	0.001

* Apgar scores are presented as median values.

† RDS, respiratory distress syndrome.

‡ Morbidity/mortality are presented as absolute numbers.

0.4 mU/mL and lipid hydroperoxide levels were 2.6 ± 0.3 nmol/mL.

Table 2 describes the characteristics of the 45 preterm infants < 32 wk gestational age who had xanthine oxidase activity and lipid hydroperoxide measurements at 1 and 4 h of age. Eighteen of them met the poor outcome criteria and 27 formed the control group. Infants with poor outcome had lower birth weights ($p = 0.02$), were less mature ($p = 0.009$), and had lower 5-min Apgar scores ($p = 0.003$) and arterial umbilical pH and base excess ($p < 0.001$) than the control group.

Figure 1 provides a summary of the plasma xanthine oxidase activity at 1 and 4 h after birth in the 45 preterm newborn infants with serial measurements. At 1 h of age, the mean xanthine oxidase activity in the group with poor outcome (2.7 ± 0.4 mU/mL) was similar to that in the control group (2.1 ± 0.3 mU/mL). At 4 h of age, the mean xanthine oxidase activity was significantly lower in the control group (1.1 ± 0.2 mU/mL) than in the group with poor outcome (4.7 ± 0.6 mU/mL, $p < 0.001$). Paired

analysis showed a significant decrease in the xanthine oxidase activity in the control group from 1 to 4 h after birth ($p = 0.004$), whereas the xanthine oxidase activity increased during this time period in the group with poor outcome ($p < 0.001$).

Plasma lipid hydroperoxide levels at 1 and 4 h after birth are shown in Figure 2. At 1 h of age, the mean lipid hydroperoxide level in the poor outcome group (2.8 ± 0.6 nmol/mL) was similar to that in the control group (2.1 ± 0.3 nmol/mL), but at 4 h of age the mean lipid hydroperoxide level was significantly lower in the control group (1.6 ± 0.2 nmol/mL) than in the group with poor outcome (4.3 ± 0.6 nmol/mL, $p < 0.001$). Paired analysis showed a significant decrease in the lipid hydroperoxide level in the control group from 1 to 4 h after birth ($p = 0.008$), whereas the lipid hydroperoxide level increased during this time period in the group with poor outcome ($p < 0.001$).

Figure 1. Mean ± SEM plasma xanthine oxidase activity in newborn infants with poor outcome ($n = 18$) and control infants ($n = 27$) at 1 and 4 h after birth. *, $p < 0.001$ vs the 1- and 4-h data of the control group and the 1-h data of the poor outcome group; **, $p = 0.004$ vs the 1-h data of the control group.

Figure 2. Mean ± SEM plasma lipid hydroperoxide levels in newborn infants with poor outcome ($n = 18$) and control infants ($n = 27$) at 1 and 4 h after birth. *, $p < 0.001$ vs the 1- and 4-h data of the control group and the 1-h data of the poor outcome group; **, $p = 0.008$ vs the 1-h data of the control group.

Xanthine oxidase activity and lipid hydroperoxide levels were not dependent on birth weight and gestational age.

DISCUSSION

The question of whether oxidant injury plays a significant role in the development of poor outcome in newborn infants is of great clinical importance, especially when preventive measures are to be instituted. Fetal antioxidant activity in rodents and rabbits remains low until late gestation (26) and antioxidant activity in cord sera of infants with birth weights ranging from 830 to 3700 g correlates with birth weight (27), indicating that preterm infants may be deficient of antioxidants and at a higher risk for oxidant injury than term infants.

Xanthine oxidase plays a pivotal role in ischemia-reperfusion injury (13). The production of free oxygen radicals by the hypoxanthine-xanthine oxidase system during reoxygenation after hypoxia may play a major role in the development of poor outcome in sick infants, as shown by elevated levels of hypoxanthine in the body fluids of preterm infants suffering or dying from severe respiratory distress syndrome (12, 19) with a positive correlation between hypoxanthine levels and the duration of hypoxia before death (19), in the plasma of infants who subsequently developed cavitating periventricular leukomalacia (28), and in some studies of cord blood of asphyxiated infants (11, 29). The latter finding was not corroborated by Lipp-Zwahlen *et al.* (30), who found a wide variation and overlap in the cord plasma hypoxanthine levels of normal infants and those with suspected intrauterine hypoxia. Plasma xanthine oxidase activity is relatively low under normal circumstances. In adult rats, plasma xanthine oxidase activity amounts to approximately 8–10 mU/mL (31, 32), and in healthy adults activity ranges from 0 to 1.20 mU/mL (24). In adult patients, circulating xanthine oxidase activity is about 2 nmol/min/mL and increases to about 6 nmol/min/mL after ischemic reperfusion (33). Tan *et al.* (16) found endogenous xanthine oxidase activity in plasma obtained at delivery from umbilical cord artery and vein to be 2.4 ± 0.5 and 2.5 ± 0.3 (mean $\pm$ SD) μ U/mL, consistently higher than in adult plasma (16). Our data on arterial cord plasma xanthine oxidase activity are similar to those of Tan *et al.* (16).

Between 1 and 4 h of age, xanthine oxidase activity decreased sharply in control infants, whereas it increased in infants with poor outcome. At 4 h of age, preterm infants who met the poor outcome criteria had significantly higher levels of plasma xanthine oxidase activity than preterm infants who did well. These results suggest that during the first hour after birth elevated levels of xanthine oxidase activity are a normal physiologic phenomenon, supporting the observation that hypoxanthine, the main substrate of xanthine oxidase, is elevated during the first 2 h after birth in about 50% of newborn infants regardless of their outcome (28). The changes in xanthine

oxidase activity over time were accompanied by similar differences in lipid hydroperoxide levels. The pronounced increase in both parameters in the poor outcome group between 1 and 4 h of age suggests a role for oxidant injury in the subsequently emerging morbidity and mortality.

However, even though preterm infants with poor outcome have a higher plasma xanthine oxidase activity compared with those with good outcome, it has not been proven that any causality exists. Vetteranta and Raivio (15) showed in human fetuses that xanthine oxidase activity decreases in the intestine toward term. Therefore, there is a possibility that the higher xanthine oxidase activity in the poor outcome group, with a lower gestational age than the good outcome group, only reflects a higher xanthine oxidase activity in the intestine. Six infants of the poor outcome group suffered from necrotizing enterocolitis *versus* none in the controls, suggesting that necrotizing enterocolitis contributes to a higher xanthine oxidase activity in plasma, maybe even long before any symptoms of necrotizing enterocolitis appear.

The decision to perform 1- and 4-h measurements of xanthine oxidase activity and lipid hydroperoxide levels was based on the observation that free oxygen radical formation peaks at 2 min after reflow and continues up to 3 h of reperfusion (34). In adult transplant patients, lipid hydroperoxide levels peak 1 h after reperfusion of transplant organ tissues and return to baseline levels in 3–4 h (35). This was not the case in our population of preterm infants with a gestational age < 32 wk. Pitkanen *et al.* (36) found that exhaled ethane and pentane levels, a measure of the degree of lipid peroxidation, are low during the first 24 h in very-low-birth-weight infants with severe respiratory distress syndrome but correlate positively with inspired oxygen concentrations after the third day of life, with a higher degree of peroxidation occurring in infants with poor outcome compared with those who survive intact. These data (36) suggest that peak lipid peroxidation occurs later than 4 h of age in preterm infants. However, these variations may also be explained by the methodologic differences in the various indices of lipid peroxidation measured.

From this study, we conclude that serial measurements of plasma xanthine oxidase activity and lipid hydroperoxides may distinguish critically ill infants destined for major morbidity and mortality. Plasma xanthine oxidase activity, and to a lesser extent plasma lipid hydroperoxide levels, at 4 h of age may be clinically useful as indicators of the severity of acute perinatal hypoxia-ischemia in preterm infants.

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