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Ferroxidases and Xanthine Oxidase in Plasma of Healthy Newborn Infants

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In the neonatal period, there is a high iron load, while both the level and molar oxidase activity of ceruloplasmin are low. On the other hand, the neonatal xanthine oxidase (XO) activity is higher than later in life and XO has a significant iron-oxidizing capacity. We therefore studied the physiological contribution of XO to the ferroxidase activity of the plasma in 20 full-term newborn infants. Ferroxidase activity was measured spectrophotometrically, with Fe^{++} as substrate. The uric acid formed by XO was assayed by means of HPLC, with electrochemical detection.

The total ferroxidase activity in the plasma was about one-fourth of the adult level and rapidly increased doubling within 3 days after birth. About 90% of the plasma ferroxidase activity was due to ceruloplasmin, the remainder being accounted for by ferroxidase II. The XO activity underwent a 30% (statistically non-significant) elevation at 24 h, though ferroxidase activity attributable to XO was not detected at any time.

Accordingly, XO does not seem to add substantially to the total iron-oxidizing capacity of the plasma in the neonatal period. The high molar ferroxidase activity is probably of importance at the endothelial cell surface.

Keywords: Ferroxidase; Ceruloplasmin; Xanthine oxidase; Newborns

INTRODUCTION

Iron plays an essential role in many cellular functions, such as oxygen transport, the mitochondrial energy metabolism and electron transport. On the other hand, non-protein bound iron also promotes free radical mediated damage to protein, lipid and DNA molecules through one-electron reduction of hydrogen peroxide and the generation of reactive hydroxyl radicals. The

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appearance of non-protein bound iron in the plasma can be induced by an iron overload, the increased generation of free radicals which liberate ferrous ion from its protein-bound form, or a decreased antioxidant defense. Its presence has been detected in the plasma of patients with hemochromatosis, thalassemia, adult respiratory distress syndrome, or rheumatoid arthritis.[1–4] Free ferrous ion has also been found in the plasma of both preterm and term babies in whom physiological hemolysis and heme degradation and the imbalance of reactive oxygen production and antioxidant defense capacity may be causal factors.[5,6]

The main defense mechanisms of the plasma against iron toxicity are the oxidation of ferrous ion to the ferric form and the binding of iron by transferrin. The ferroxidase activity of the plasma is mainly due to the copper-containing α_2-glycoprotein ceruloplasmin.[7] By virtue of its ability to catalyze the oxidation of ferrous ions, ceruloplasmin inhibits both lipid peroxidation and the Fenton reaction.[8] Moreover, it accelerates the binding of ferric ion to transferrin, suggesting a coordinated action of the two proteins.[9] Transferrin–iron is then transported from the plasma to the intracellular space.[10] In addition to its antioxidant action, the ferroxidase activity of ceruloplasmin seems to play an essential role in the mobilization of iron from hepatocytes and reticuloendothelial cells.[11,12]

Besides ceruloplasmin, the other substances with an iron-oxidizing capacity in the plasma include ferroxidase II and xanthine oxidase (XO). The activity of the azide-resistant ferroxidase II has been suggested to be due either to an enzymic activity of low density lipoprotein or to lipid peroxides.[13,14] In healthy adults, it accounts for 5–10% of the total ferroxidase activity in the plasma.

XO catalyzes the oxidation of several substrates including hypoxanthine and xanthine, but it also possesses a significant iron-oxidizing activity. The molar ferroxidase activity of intestinal XO has been found to be 1000 times higher than that of ceruloplasmin.[15] Iron oxidation by XO is not inhibited by superoxide dismutase or catalase; thus the reactive oxygen species produced during the oxidation of purines are not involved. XO also promotes the incorporation of iron into apotransferrin and apoferritin.[15,16] These actions have been implicated in iron absorption by intestinal mucosa cells.

In the first few days after birth, there is a high iron load, while both the level and the molar oxidase activity of ceruloplasmin are low.[17,18] These latter reach adult values only between 3 and 6 months after birth.[19,20] On the other hand the neonatal XO activity is higher than later in life.[21] These data prompted us to study the possible contribution of XO to the iron-oxidizing activity of plasma in the neonatal period. This question also seemed to be of interest because allopurinol, an inhibitor of XO, has been used in some clinical trials to prevent free radical-mediated injuries in newborns.[22–24]

PATIENTS, MATERIALS AND METHODS

Patients

Venous blood samples were collected from 20 healthy newborns (gestational age: 38.92 ± 0.86 weeks, weight: 3431 ± 425 g) immediately after birth (cord blood), at 24 h and at 3 days after delivery. The investigation was part of a study involving a search for laboratory parameters of prognostic significance in the neonatal period. The study was approved by the Human Investigation Review Board of the University and informed parental consent was obtained before the collection of blood samples. Adult plasma ferroxidase levels were determined in 28 healthy volunteers (age 41.37 ± 10.34 years).

Materials

Catalase (H_2O_2:H_2O_2 oxidoreductase, EC 1.11.1.6), superoxide dismutase (superoxide:superoxide

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oxidoreductase, EC 1.15.1.1), dithioerythritol (DTE) and conalbumin were purchased from Sigma Chemical Co. (St. Louis, USA). Phenylmethanesulfonyl fluoride (PMSF) and Sephadex G-25 Fine were from Fluka Ag (Buchs, Switzerland) and Pharmacia Biotech (Uppsala, Sweden), respectively. All other chemicals were of reagent grade.

Ferroxidase Activity

Ferroxidase activity was determined via the oxidative incorporation of ferrous ion into apo-ovotransferrin by a modification of the method of Johnson *et al.*[25] Heparinized plasma samples stored at −72°C for no longer than 2 weeks were used for the analysis. A 20 μl aliquot of plasma was incubated at 37°C in the presence of 120 μM ferrous ammonium sulfate, 0.5% (w/v) apo-ovotransferrin and 0.52 M sodium acetate buffer (pH 6.5). The formation of the complex was followed spectrophotometrically at 460 nm. One unit of ferroxidase catalyzes the transformation of 1 μmol Fe^{++} to Fe^{+++} per minute under the specified conditions. The assay measures plasma total ferroxidase activity. Contributions by ceruloplasmin and XO were determined by preincubation (10 min) of the samples with 1 mM azide or 1 mM allopurinol, respectively. Ferroxidase activity was also measured after the addition of xanthine to the assay mixture in final concentrations of 15–50 μM.

Xanthine Oxidase Activity

XO activity was measured via the formation of uric acid from xanthine by combining previously accepted standard methods. Blood was collected in heparinized tubes, placed on ice and immediately mixed with a stabilizing solution containing 10 mM DTE, 1 mM PMSF and 1 mM ethylenediaminetetraacetic acid (EDTA) in a 50 mM phosphate buffer, pH 7.8. After separation of the blood (1500 *g*, 15 min at 4°C), 100 μl aliquots of plasma samples were passed over a Sephadex G-25 column to remove endogenous substrates and low molecular weight inhibitors.[26] The effluents were supplemented with superoxide dismutase (60 U/ml) and catalase (200 U/ml) to give the respective final concentrations, and incubated at 37°C for 60 min in the presence or absence of xanthine (150 μM).[27] Controls containing allopurinol (100 μM) were run with each sample. Reactions were stopped by adding three volumes of cold acetonitrile. The samples were centrifuged and the supernatant was evaporated. The residue from evaporation was resuspended in the mobile phase (HPLC) and XO activity was assayed by quantifying the uric acid formation with HPLC (column: Spherisorb ODS 2 (5 μm packing) 40×250 mm (Pharmacia LKB, Uppsala, Sweden), mobile phase: 50 mM sodium acetate buffer, pH 4.75 with 0.1 mM EDTA, flow rate: 1 ml/min) and electrochemical detection (Pharmacia LKB, oxidation potential +0.76 V).[13] One unit of activity was defined as the amount of enzyme required to

TABLE I Plasma ferroxidase and XO activities in the first 3 days of life in healthy term newborns (data represent the mean±SEM ($n = 20$). (ND: not detectable)

	0 h	*24 h*	*72 h*
Total ferroxidase activity (mU/ml)	208±15	281±19*	421±24*
Ceruloplasmin	192±16	255±20†	381±24*
Ferroxidase II	16±3	25±3†	41±5*
XO	ND	ND	ND
XO activity (μU/ml)	102±11	132±15	120±15

* $p<0.001$ vs. the earlier value of the same type of activity.
† $p<0.01$ vs. the earlier value of the same type of activity.

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produce 1 μmol uric acid per min under the specified conditions.

Statistical Analysis

Data were expressed as mean ± standard error of the mean ($x \pm$ SEM). The Kruskall–Wallis test was used, followed by the Mann–Whitney method, to determine significant differences over time within the parameters. The associations between the different types of ferroxidase activity and XO activity were determined by multiple regression analysis. Statistical significance was defined as $p < 0.05$.

RESULTS

Ferroxidase Activities

The plasma total ferroxidase, ceruloplasmin and ferroxidase II activities displayed significant, time-related increases during the observation period (Table I). However, ferroxidase activity inhibited by allopurinol was not detected in any of the plasma samples. The addition of xanthine to the assay mixture had no influence on the results.

The total ferroxidase, ceruloplasmin and ferroxidase II activities in the plasma of the healthy adults were 748 ± 28, 706 ± 27 and 41 ± 7 mU/ml, respectively. Swain *et al.*[28] reported that the serum ferroxidase activity in healthy subjects was 780 ± 75 U/l, while Erel[29] found the activity to be 537 ± 201 U/l by an automated measurement method.

Xanthine Oxidase

The plasma XO activity was slightly but not significantly (by 30%) elevated at 24 h as compared with the value at birth, and remained at this elevated level during the observation period (Table I).

The total ferroxidase and ceruloplasmin activities correlated strongly and positively at each measurement (0 h: $r = 0.9425$, $p<0.001$; 24 h: $r = 0.9812$, $p<0.001$ and 72 h: $r = 0.9724$, $p<0.001$). Ferroxidase II and XO did not correlate significantly with any of the other tested parameters.

DISCUSSION

The total ferroxidase and ceruloplasmin activities measured immediately after birth in healthy term newborns were only about one-fourth of the values observed in adults. However, the plasma ferroxidase activities increased rapidly during the observation period doubling within 3 days. Almost 90% of the ferroxidase activity was due to ceruloplasmin, while the remainder was accounted for by ferroxidase II. The ratio of the different ferroxidases proved to be constant in time. The higher contribution of ferroxidase II to the plasma iron-oxidizing capacity as compared with that in adults (approximately 8–10% vs. 5%) resulted from the low ceruloplasmin level in the infants.

To be able to assay non-ceruloplasmin ferroxidases as well, we used the method of Johnson *et al.*, applying Fe^{++} as substrate.[25] The results correlated well with data obtained with the colorimetric p-phenylenediamine assay, both showing an increase in the oxidase activity of ceruloplasmin after birth.[30] In most studies relating to newborns, the concentration but not the activity of ceruloplasmin was measured, and the observations were somewhat controversial: both rising and steady levels in the postnatal period have been reported.[31,32] For assessment of the iron-oxidizing capacity, enzymatic measurement of ceruloplasmin activity may be preferred to immunochemical techniques. The reason is that the level and molar activity of ceruloplasmin are not strictly correlated, but may change depending on the age, the occurrence of disease or conditions influencing the copper status. Mora *et al.* observed that ceruloplasmin in

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newborn serum has a lower oxidase activity per molecule than that in adult serum.[17] Perlman *et al.* found discordant changes between the ceruloplasmin measured by immundiffusion and the enzyme assay between 7 and 11 weeks after birth.[33] Further, Powers *et al.*[34] reported that a high vitamin C level may inhibit the iron-oxidizing activity of ceruloplasmin in premature babies, though other studies seem to contradict this finding.[35,36]

While the plasma of healthy newborns exhibited both ferroxidase I (ceruloplasmin) and ferroxidase II activities, the inhibition of ferroxidase activity by allopurinol was never detected. Topham *et al.* found xanthine to be essential for the formation of Fe^{+++}–transferrin by XO.[15] The K_M values determined for xanthine and hypoxanthine were 5–7 μM. Newborn infants exhibit an augmented hypoxanthine level, the mean value during the first 12–36 h of life in full-term babies being 5.5 μM.[37] Accordingly, the endogenous level of hypoxanthine should be sufficient to yield appreciable rates of Fe^{+++}–transferrin formation. The ferroxidase activity of the plasma samples was also tested after the addition of exogenous xanthine, though this alteration in the assay system did not affect the results. The observation that XO promotes the oxidative incorporation of iron into transferrin was made on enzyme isolated from intestinal mucosa cells. However, the iron-oxidizing capacity is not limited solely to this form of the enzyme: ferroxidase activity attributable to XO has also been demonstrated in various aneurysms and endarterectomies.[38] In the latter study, the samples also exhibited high purine oxidase activities. The molar xanthine and iron oxidase activities of XO are of the same order of magnitude.[15] Thus, the low measure of xanthine oxidation by XO observed in the present experiments is in accordance with the low ferroxidase activity (undetectable by the applied method).

The values reported for the xanthine-oxidizing activity of XO in human plasma vary greatly. However, the studies agree that certain conditions such as ischemia/reperfusion lead to significant rise in the circulating enzyme level.[21,39–41] In the present study, healthy newborns displayed approximately twice the level previously observed in adults.[42] Tan *et al.* also reported a higher xanthine-oxidizing activity in plasma obtained at delivery than that in adult plasma.[21] From a comparison of term and preterm newborn infants Supnet *et al.* found similar enzyme activities in the plasma of healthy infants at gestational weeks 27–39.[43]

During the first 3 days after birth, XO underwent only slight alterations in the term newborns. These slow and moderate changes differ from the rapid and marked changes demonstrated in premature infants by Supnet *et al.*[43] They reported a significant elevation in XO activity in premature infants with a poor outcome, and a decrease to approximately half of the starting activity in premature controls between 1 and 4 h after birth. In the circulation, XO has a very long circulating half-life, approximately 4–6 h.[44] Rapid changes probably result from alterations in the vascular distribution of the enzyme. XO is in equilibrium between the plasma phase and the endothelial cell surface.[45,46] Accordingly, after release of the intracellular enzyme (e.g. due to hypoxia during birth), the increase in plasma activity is possibly limited by the binding of the circulating enzyme to the endothelial cells. The XO activity at the cell surface was earlier suggested to be considerably higher than that measured in the plasma.[40] Thus, a moderate rise in plasma XO activity, as observed in the present study, may reflect a significant enzyme release and a considerable increase in the total vascular (circulating and cell-bound) enzyme activity.

Though XO does not seem to add substantially to the total iron-oxidizing capacity of the plasma, its high molar ferroxidase activity may be of importance at the endothelial cell surface. The superoxide anion and hydrogen peroxide produced by the enzyme have been implicated in the

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regulation of neutrophil adherence and the vascular response.[46,47] On the other hand, the reaction of the same oxygen species with ferrous ions may lead to a toxic effect through formation of hydroxyl radicals. XO may potentially hinder this latter reaction by oxidizing ferrous ion to the less reactive ferric form. Beside the capability of uric acid to scavenge hydroxyl radicals, the ferroxidase activity of XO might be another important factor, which reduces the hazard of reactive oxygen production by the enzyme and promotes the regulatory role of its products.

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