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Recycling of Glutathione during Oxidative Stress in Erythrocytes of the Newborn

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ABSTRACT. The ability of erythrocytes from newborn babies and adults to maintain reduced glutathione levels during oxidative stress was studied. *In vitro* incubation of erythrocytes with H_2O_2, with or without inactivation of catalase, caused a rapid depletion of reduced glutathione (GSH) and concomitant accumulation of oxidized glutathione followed by recovery of GSH and fall of oxidized glutathione to initial values in all subjects. Inactivation of catalase resulted in a 50% loss of intracellular glutathione ($p < 0.005$), a larger maximum GSH depletion ($p < 0.05$), and a longer GSH recovery time ($p < 0.005$). Erythrocytes from newborn babies showed a smaller maximum GSH depletion ($p < 0.05$) and a shorter GSH recovery time ($p < 0.005$) compared with those from adults. These differences between the newborn and adult groups persisted after inactivation of catalase. An increase in maximum GSH depletion and GSH recovery time ($p < 0.005$) was observed when a lower hematocrit was used for these GSH recovery studies. Effective glutathione recycling in erythrocytes may protect immature tissues of the newborn baby from peroxidative damage. (*Pediatr Res* 32: 399–402, 1992)

Abbreviations

GSH, reduced glutathione
GSSG, oxidized glutathione

In vitro and *in vivo* studies have shown that the erythrocytes of adult animals can protect other tissues, *e.g.* lung, against damage induced by reactive oxygen species (1). Erythrocytes can catabolize an H_2O_2 load produced by leukocytes (2) and have been shown to protect both cultured pulmonary endothelial cells (3) and the whole lung (4, 5) from oxidative damage. The action of catalase and the glutathione recycling system is believed to be the key factor in this protective mechanism (1).

The preterm baby has poorly developed antioxidant enzyme systems in various organs, *e.g.* lung, and is susceptible to oxygen-induced tissue damage, *e.g.* bronchopulmonary dysplasia (6, 7). Thus, the ability of the erythrocyte to catabolize H_2O_2 may be of particular importance in these patients. The ability of the erythrocyte to handle oxidative stress induced by H_2O_2 can be assessed *in vitro* by serially measuring the initial fall of GSH and the concomitant rise of GSSG and the subsequent recovery of GSH and fall of GSSG to initial values. Glutathione recycling has already been used to assess the antioxidant capacity of erythrocytes of adults (8) and cell cultures (9, 10). We compared glutathione recycling during exposure to H_2O_2 in erythrocytes of newborn babies and adults. The erythrocyte activities of enzymes responsible for H_2O_2 catabolism, *i.e.* catalase, glutathione peroxidase, and glutathione reductase, were also measured.

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MATERIALS AND METHODS

This study was approved by the Scientific Committee of the Department of Pediatrics and the Ethical Committee of the University Hospital of Leiden.

Patients. Umbilical cord blood samples were obtained from eight preterm and nine term infants {gestational age [mean (SD)] 32.9 (2.4) and 40.2 (1.5) wk, respectively} within 15 min after delivery of the placenta. The babies were of normal birth weight (10th–90th percentile) and showed no signs of birth asphyxia (Apgar score 1 min ≥ 9, umbilical vein pH ≥ 7.25), respiratory distress, or infection. Their mothers, healthy Caucasian nonsmokers (3), did not receive vitamin or iron supplements. Venous blood samples were taken from 10 healthy Caucasian volunteers {age [mean (SD)] 24.9 (2.4) y}, who were nonsmokers.

From an additional group of 10 healthy term infants {gestational age [mean (SD) 40.6 (1.4) wk, other characteristics as above} umbilical cord blood samples were obtained to study *in vitro* the influence of the hematocrit on glutathione recycling.

Procedure. Blood was gently withdrawn (1.1-mm diameter needle) into heparinized tubes and immediately centrifuged (750 × *g*, 10 min). Plasma and buffy coat were discarded and erythrocytes were washed twice in PBS (500 × *g*, 10 min). Aliquots of packed cells diluted in PBS (40×) with or without 1 mM added sodium azide (a catalase inhibitor) were incubated with H_2O_2 (400 μM) and glucose (5 mM) in a shaking water bath (37°C) for 30 min. Control incubations were performed in the absence of H_2O_2. Samples were withdrawn at specified time intervals and, after preparation, stored at −20°C until spectrophotometric flow injection analysis of total glutathione (GSH + GSSG) and GSSG (11). Initial GSH (total glutathione minus GSSG) and GSSG concentrations were calculated using a GSSG standard (11) and expressed as μmol/g Hb. To analyze changes with time during incubation with H_2O_2, the GSH level was expressed as the percentage of total glutathione. The following parameters were then calculated (Fig. 1): maximum GSH depletion, *i.e.* initial GSH% minus lowest GSH%; GSH depletion time, *i.e.* time at which maximum GSH depletion occurred; and GSH recovery time, *i.e.* time at which GSH recovered to 80% of its initial value. In preliminary studies, the occurrence of membrane lipid peroxidation during glutathione recycling was assessed by measuring thiobarbituric acid reactive substances in newborn and adult erythrocytes. In the absence of azide, no lipid peroxidation was observed and in the presence of azide a small increase in thiobarbituric acid reactive substances occurred, but this did not differ between the two patient groups.

The influence of the hematocrit on glutathione recycling was studied *in vitro*. A dilution of 40× (see above) served as a control and a dilution of 30× (high hematocrit) and 80× (low hematocrit)

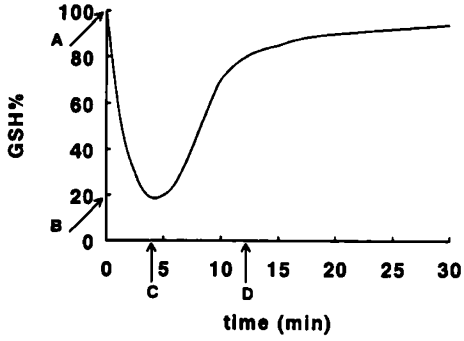

Fig. 1. The changes in GSH% induced by incubation of erythrocytes with H_2O_2. GSH parameters were measured as follows: *1*) maximum GSH depletion, *i.e.* initial GSH% (*point A*) minus lowest GSH% (*point B*); *2*) GSH depletion time, *i.e.* time at which maximum GSH depletion occurred (*point C*); and *3*) GSH recovery time, *i.e.* time at which GSH recovered to 80% of its initial value (*point D*).

as a model for polycythemia and anemia, respectively. Incubations without sodium azide were performed as described above.

Enzymes. Activities of catalase (EC 1.11.1.6), glutathione peroxidase (EC 1.11.1.9), and glutathione reductase (EC 1.6.4.2) were measured in erythrocyte hemolysates using standard spectrophotometric techniques (12–14).

Reagents. All reagents were of the best grade commercially available. H_2O_2 and sodium azide pro analysi were obtained from Merck (Darmstadt, Germany) and GSSG (grade IV) was obtained from Sigma (St. Louis, MO).

Statistics. All results are reported as mean and SD or median and range. The SPSS-PC$^+$ software (version 3.0) was used for statistical analysis. Differences in maximum GSH depletion between the preterm, term, and adult groups were tested by pair-wise comparisons using the Mann-Whitney test; the influence of azide on this parameter within each of the groups was tested using the Wilcoxon test. Differences in maximum GSH depletion produced by altering the hematocrit were tested by pair-wise comparisons using the Wilcoxon test. For all other variables, the unpaired and paired *t* test were used. The correlation between gestational age and GSH recovery time was tested by Pearson's method. Values of $p < 0.05$ were regarded as significant.

RESULTS

Initial erythrocyte GSH concentrations were higher in the preterm and term babies than in adults [12.0 (2.6), 13.0 (4.6), and 9.6 (2.0) μmol/g Hb, respectively, $p < 0.05$]. The initial GSH% of the total glutathione concentration was similar in all groups [99.2 (1.1)%]. In the newborn babies, catalase and glutathione peroxidase activities were lower ($p < 0.05$) and glutathione reductase activity was higher ($p < 0.05$) than in the adults (results not shown).

The total glutathione level was stable during control incubations and incubations with H_2O_2 without azide in all groups. However, in the presence of H_2O_2 and azide, total glutathione gradually decreased to 50% of the initial value after incubation for 30 min ($p < 0.005$). This decline did not differ between the three groups.

GSH% and GSSG% were stable during control incubations. However, in the presence of H_2O_2 with or without azide, GSH% and GSSG% changed significantly; immediately after H_2O_2 addition, GSH% fell with a concomitant increase in GSSG%, followed by a gradual recovery of GSH% to its initial high level and return of GSSG% to its initial low level. Figure 2 shows the serial changes in GSH% with and without azide in the three groups. Within all groups, inactivation of catalase produced a larger maximum GSH depletion ($p < 0.05$) and a longer GSH depletion time ($p < 0.05$) and GSH recovery time ($p < 0.005$). In the absence of azide, erythrocytes of newborn babies showed

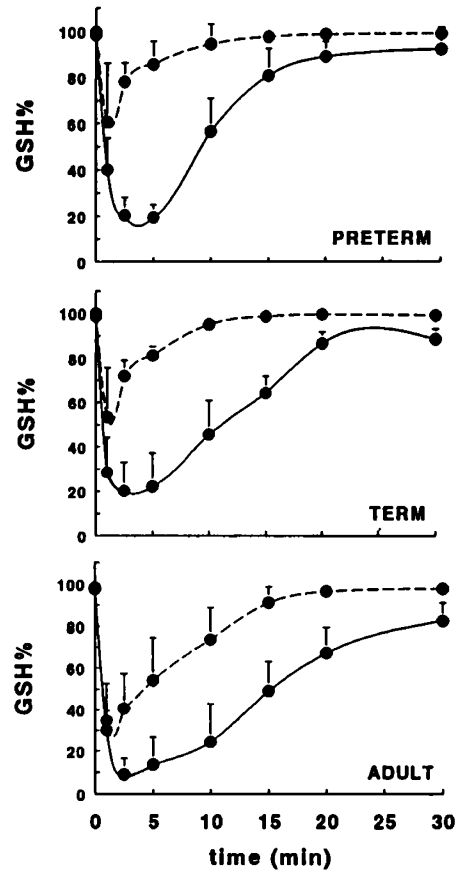

Fig. 2. Changes in GSH% (mean, SD) in the preterm, term, and adult groups in erythrocytes incubated with H_2O_2 in the absence (- - - -) and the presence (——) of azide.

Table 1. *Influence of hematocrit on GSH parameters in cord blood erythrocytes incubated with H_2O_2 without addition of azide*

	High hematocrit	Control	Low hematocrit
Maximum GSH depletion (%)*	31.8 [18.1–46.8]	39.9 [25.0–49.5]	57.0† [45.2–64.1]
GSH depletion time (min)‡	1.3 (0.2)	1.3 (0.1)	1.4 (0.1)
GSH recovery time (min)‡	2.3 (1.0)	2.6 (0.6)	5.1† (1.0)

* Median [range].
† $p < 0.005$ compared with control.
‡ Mean (SD).

a smaller maximum GSH depletion ($p < 0.05$) and a shorter GSH recovery time ($p < 0.005$) but no difference in GSH depletion time compared to those of adults. These differences persisted after inactivation of catalase. However, in the newborn group, the preterm babies had a shorter GSH recovery time than the term babies ($p < 0.01$) and GSH recovery time correlated with gestational age ($r = 0.68$, $p < 0.02$, $n = 12$).

Table 1 shows the influence of erythrocyte concentration on the GSH parameters. The smaller maximum GSH depletion and shorter GSH recovery time in the "high hematocrit" samples compared to the control samples were not statistically significant. However, there was a significantly greater maximum GSH depletion and longer GSH recovery time in the "low hematocrit" samples ($p < 0.005$). The hematocrit had no influence on GSH depletion time.

DISCUSSION

Erythrocytes are exposed to high oxygen concentrations and possess powerful antioxidant defenses. An important oxidant in the blood is H_2O_2, which is produced physiologically, *e.g.* by erythrocytes (15), and pathologically, *e.g.* by activated polymorphonuclear leukocytes (16). Its catabolism by erythrocyte catalase and glutathione peroxidase is important in protecting not only the erythrocyte, but also other tissues, *e.g.* lung (1, 3–5). Catalase needs no additional cofactor for H_2O_2 catabolism. However, glutathione peroxidase requires GSH as a substrate and glutathione reductase-mediated reduction of GSSG recycles GSH, maintaining it at a high concentration. This glutathione recycling during oxidative stress has been studied in cell cultures (9, 10) and in erythrocytes of adults (8). However, in erythrocytes of the newborn, although the H_2O_2-induced fall of GSH has been studied (17, 18), the characteristics of the recovery of GSH have not been studied previously.

Our results provide further evidence that catalase plays an important role in handling an H_2O_2 load in the erythrocyte (1, 19, 20). Inactivation of catalase by the addition of azide had a marked influence on both total glutathione and GSH. The glutathione recycling system was exposed to greater oxidative stress and larger amounts of GSSG were formed. It has been suggested that the GSSG can be lost as a result of formation of mixed disulfides with Hb (9) and active transport out of the cell (9, 21). These mechanisms could explain the 50% loss of total glutathione in our studies. The remaining glutathione was still recycled, although a greater maximum GSH depletion and a longer GSH recovery time occurred in all groups. In the newborn group, the preterm babies now had a shorter GSH recovery time than the term babies.

Our results confirm the lower catalase and glutathione peroxidase levels and the higher glutathione reductase and GSH levels in erythrocytes of newborn babies compared with those of adults (18, 22–24). These findings could be due to the raised reticulocyte count typically present in cord blood. However, adults with a reticulocyte count similar to the newborn show an increased glutathione peroxidase activity and no increase in GSH level (22). Furthermore, in adults with a greatly increased reticulocyte count, the glutathione reductase activity is still much lower than that in newborn babies (25). The regulation of expression of the enzymes involved in glutathione recycling appears to be different in fetal erythrocytes than in those from adults (22, 25). This regulation appears to change gradually: in subjects aged 1 mo to 63 y, glutathione peroxidase activity correlated positively and glutathione reductase activity negatively with age (26).

It has been argued that the smaller fall in GSH levels during oxidative stress in erythrocytes of newborn babies compared to adults is due to the decreased glutathione peroxidase level resulting in an inability to utilize GSH (27). However, it is well recognized that an enzyme concentration measured in a hemolysate may not reflect its actual activity in the intact erythrocyte (18, 28). Glader and Conrad (18) showed that exposure of erythrocytes to H_2O_2 stress after inhibiting catalase and glutathione reductase activities resulted in the oxidation of similar amounts of GSH in erythrocytes of newborn babies and adults. This demonstrated that the lower erythrocyte glutathione peroxidase level of newborn babies was not functionally significant (18). Our findings support this conclusion: if the glutathione peroxidase activity was limited, maximum GSH depletion would have occurred later than in erythrocytes of adults, and a greater H_2O_2 load, due to inactivation of catalase, would not have produced a greater maximum GSH depletion. It is interesting that the raised glutathione reductase level in erythrocytes of newborn babies has not been used to explain the GSH levels during oxidative stress. We suggest that the decreased fall in GSH levels recorded in other studies (18, 27) may not have been due to lack of oxidation to GSSG because of decreased glutathione peroxidase activity, but rather due to rapid reduction of GSSG back to GSH because of increased glutathione reductase activity. This interpretation is not supported by a study using a nonphysiologic oxidant: a raised glutathione reductase activity in erythrocytes of newborn babies did not result in a shorter GSH recovery time (28). These different results may be explained by differences in the oxidant used and the patient populations studied [no details of the babies' gestational age, nutritional state, and condition at birth were given (28)].

An alternative explanation for the more efficient glutathione recycling in erythrocytes of newborn babies could be that their erythrocyte membranes were more susceptible to H_2O_2 damage (17, 29, 30). Preferential targeting of these membranes might reduce the H_2O_2 load on their glutathione recycling system, resulting in a smaller GSH depletion and a shorter GSH recovery time. However, this explanation is not likely because we used a much lower H_2O_2 concentration than is used to study the susceptibility of erythrocyte membranes to lipid peroxidation, *i.e* 400 μM *versus* 10 mM (31) or 110 mM (32). As discussed in Materials and Methods, this low H_2O_2 concentration produced no differences in lipid peroxidation between babies and adults. These findings support the contention that the glutathione recycling system of neonatal erythrocytes was subjected to an H_2O_2 load equal to that of the adult erythrocytes but was able to more efficiently catabolize the H_2O_2.

Clinical implications. At birth, newborn babies have a relative polycythemia and their erythrocytes have a higher GSH concentration and a more efficient glutathione recycling system than adults. This effective system for H_2O_2 catabolism may help protect against oxygen toxicity by compensating for the deficient antioxidant capacity in other tissues, *e.g.* lung (1, 7). The efficiency of this system could be influenced postnatally by quantitative and qualitative changes in the erythrocytes. It has been suggested that transfusions or exchange transfusions may predispose babies to oxygen toxicity because of a greater oxygen dissociation from adult erythrocytes (33). However, the results from this study and our previous study on plasma antioxidants (34) suggest that the influence of transfusions on oxygen toxicity is complex and not always disadvantageous. Transfusion of an anemic baby could improve its total antioxidant capacity by increasing the contribution of the erythrocytes and plasma. Selenium and riboflavin are cofactors of glutathione peroxidase and glutathione reductase, respectively, and their bioavailability could be influenced by the nutrition and therapy of the baby. For example, human milk contains more selenium than preterm formula (35) and phototherapy can decrease plasma riboflavin levels (36).

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