0031-3998/10/6704-0407
PEDIATRIC RESEARCH

Vol. 67, No. 4, 2010
Printed in U.S.A.

Glutathione Synthesis Rates in Early Postnatal Life

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ABSTRACT: Preterm infants have diminished antioxidant defenses. Glutathione (GSH), the main intracellular antioxidant, increases upon amino acid (AA) administration in preterm infants, without an accompanying rise of the fractional synthesis rate of GSH (FSR_{GSH}) This study investigated the mechanism behind this increased GSH concentration by determining GSH synthesis in the first days after birth using stable isotope techniques in very low-birth-weight (VLBW) infants receiving i.v. AAs. Advanced oxidized protein products (AOPPs) were determined to quantify oxidative stress. Eighteen infants (birth weight 989 ± 241 g, gestational age of $27^6/_7 \pm 1^4/_7$ weeks) were studied either on postnatal day 1 or 2 (7 or 31 h postnatally, respectively). Concentration of GSH increased with postnatal age (1.45 ± 0.48 mM *versus* 1.99 ± 0.40 mM, $p = 0.019$). FSR_{GSH} was not significantly different, but the absolute synthesis rate of GSH (ASR_{GSH}) tended to be higher in the infants studied on day 2 [8.1 ± 2.7 mg/(kg · d) *versus* 10.6 ± 2.4 mg/(kg · d), $p = 0.054$]. AOPP concentrations were not different between groups. In conclusion, GSH concentration in VLBW infants increases significantly after birth. A concomitant increased synthesis rate was not found, suggesting that GSH consumption decreases upon AA administration. (***Pediatr Res* 67: 407–411, 2010**)

The sudden increase in oxygen pressure accompanying birth results in increased formation of reactive oxygen species (ROS). If birth is marked by a period of ischemia followed by reoxygenation (*e.g.* asphyxia), ROS formation is further augmented through the hypoxanthine-xanthine oxidase pathway (1,2). In addition, preterm infants are frequently exposed to ventilation with high concentrations of oxygen, further adding up to this ROS formation (3). Therefore, newborn infants, and especially preterm infants, are exposed to increased levels of ROS.

In term infants, antioxidant defenses are sufficiently present at birth to counteract this hyperoxic challenge, because antioxidant enzymes mature during late gestation (4). Several weeks before birth, parallel with the rapid rise in lung surfactant, there is a 150–200% increase in superoxide dismutase and glutathione peroxidase (5,6). In addition, the transfer of several antioxidants across the placenta increases during the last days of pregnancy (4). Thus, when an infant is born premature, most of its antioxidant defense mechanisms function suboptimally at birth. The resulting redox imbalance promotes oxidative stress, which is thought to be instrumental in the pathogenesis of the so-called "Oxygen Radical Disease in Neonatology." The latter comprises diseases such as bronchopulmonary dysplasia (BPD) and periventricular leukomalacia (PVL) (7).

Glutathione (GSH) is the most important intracellular antioxidant. GSH is a tripeptide consisting of the amino acids (AA) glutamate, cysteine, and glycine. It is synthesized in virtually every tissue but is mainly produced in liver and erythrocytes with erythrocytic concentrations being in the millimolar range (8). Erythrocytes are suggested to function as antioxidant defense by being a physiologic source of GSH and by taking up ROS (9,10).

Although preterm infants show diminished availability of other components of the antioxidant defense systems, the GSH concentrations in cord blood of preterm infants at birth exceed those of term infants (9,11,12). GSH concentrations, however, fall rapidly after birth in preterm infants. Recently, we demonstrated that administering AAs to preterm infants from birth onward results in higher GSH concentrations on day 2, compared with levels found in infants receiving glucose only (13). The purpose of this study was to reveal the mechanism behind the increased availability of GSH with early administration of AAs. We, therefore, hypothesized that GSH synthesis is already up-regulated very shortly after birth, resulting in the increased GSH concentration on postnatal day 2. In this study, we conducted a stable isotope technique to determine glutathione synthesis rates in the first days after birth in infants receiving AAs directly after birth. With stable isotope techniques, we are able to calculate the fractional synthesis rate (FSR_{GSH}), which is the fraction of the total intraerythrocytic GSH pool that is renewed per unit of time, and the absolute synthesis rate (ASR_{GSH}), which is the absolute amount of GSH that is produced per unit of time.

In addition, we quantified oxidative stress by determining concentrations of the advanced oxidized protein products (AOPP), first described as a marker for protein oxidation in uremic patients (14). Hypoxic preterm infants showed higher plasma concentrations of AOPP than normoxic preterm infants and concentrations correlated with plasma levels of hypoxanthine, which is considered a reliable marker of oxidative stress (15).

Received July 10, 2009; accepted December 15, 2009.
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Supported by Sophia Foundation for Scientific Research, Rotterdam, The Netherlands.

Abbreviations: AA, amino acid; **AOPP,** advanced oxidized protein products; **ASR,** absolute synthesis rate; **FSR,** fractional synthesis rate; **GSH,** glutathione; **ROS,** reactive oxygen species

SUBJECTS AND METHODS

This study was designed as a prospective observational clinical trial in which infants were randomized to be studied either on postnatal day 1 (early group) or 2 (late group). Because this study was performed on subsequent days, different infants were used for the tracer infusion study either early or late after birth, because of the required washout time of [1-^{13}C]glycine.

The study was performed at the neonatal intensive care unit of the Erasmus MC–Sophia Children's Hospital, Rotterdam, the Netherlands. The study was investigator initiated with no funding from industry. The Erasmus MC Medical Ethical Review Board approved the protocol, and informed written parental consent was obtained before the study.

Subjects. Preterm infants with a birth weight <1500 g, who had an indwelling arterial catheter for blood sampling and who were completely dependent on parenteral nutrition for the first 2 d of life, were eligible for this study. Exclusion criteria included receiving erythrocyte transfusions during or before the study; known congenital abnormalities; chromosome defects; and metabolic, endocrine, renal, or hepatic disorders.

Patients received glucose and 2.4 g of AA/(kg · d) (Primene 10%, Baxter, Clintec Benelux N.V., Brussels, Belgium) i.v., starting within 2 h after birth. AAs and glucose solutions were infused constantly without interruptions during the study. The AA solutions did not contain riboflavins, which are known to generate H_2O_2 when exposed to light (16). Lipids were not administered until the end of the study period.

Birth weight, gestational age, birth weight *Z*-scores, antenatal corticosteroid usage, and severity of illness at entry of the study by means of Apgar and CRIB (clinical risk index for babies) scores (17) were recorded for all infants. Furthermore, blood gases, plasma AA concentrations, dependence on supplemental oxygen [expressed as the median (min–max) FiO_2 from birth until the end of the study], caloric intake, and AA intake were recorded.

Tracer infusion protocol. Patients received a primed (40 μmol/kg), continuous [20 μmol/(kg · h)] infusion of [1-^{13}C]glycine during 6 h either on day 1 (early) or on day 2 (late). [1-^{13}C]Glycine (99% enriched, sterility and pyrogenicity tested) was purchased from Cambridge Isotope Laboratories (Andover, MA) and was diluted with a 0.9% saline solution by the hospital's pharmacy after which it was again tested for sterility and pyrogenicity. Tracers were infused with a Perfusor fm infusion pump (B. Braun Medical B.V., Oss, the Netherlands) along the same infusion route as the parenterally administered nutrients. Blood was sampled from an indwelling arterial catheter after 4, 5, and 6 h and collected in microtainers containing EDTA to quantify FSR_{GSH} in erythrocytes.

Sample analyses. Blood samples were immediately put on melting ice, after which they were centrifuged at 3500 × g for 10 min at 4°C. The plasma fraction was removed and stored separately for measurement of oxidative stress markers. The lower layer, containing primarily erythrocytes, was reconstituted to its original volume with ice-cold distilled water. The plasma fraction and cell fraction were subsequently stored at −80°C until further analysis.

Glutathione enrichments and concentration. Enrichments of GSH and its precursor glycine as well as GSH concentrations were determined to calculate fractional and absolute synthesis rates. For this purpose, a recently described technique, based on a LC-Isolink interface (Thermo Electron, Bremen, Germany) coupled to a Delta XP isotope ratio mass spectrometer (Thermo Electron, Bremen, Germany) (LC-IRMS), was used (18). This highly sensitive method requires only a very small sample volume and does not require derivatization.

The concentration of GSH in human blood erythrocytes was measured by LC-IRMS using an internal standard method as described previously (18).

Calculations. FSR_{GSH} represents the fraction of the total intraerythrocytic GSH pool that is renewed per unit of time and is expressed as %/d.

$$FSR_{GSH}\ (\%/d) = \frac{\text{slope}E_{[1-^{13}C]GSH_{14,5,6}}}{E_{\text{intraerythrocytic}[1-^{13}C]\text{glycine}}} \times 24\ \text{h} \times 100\%$$

where *E* stands for enrichment expressed as mole percent excess (MPE). The numerator (product) of this equation represents the hourly increase in [1-^{13}C]glycine bound GSH as calculated from the increase in enrichment between 4 and 6 h of infusion. The denominator (precursor) represents the intraerythrocytic 1-^{13}C enrichment of free glycine at isotopic steady state. A steady-state plateau was defined as an insignificant change in time in intraerythrocytic enrichment.

Subsequently, the intravascular ASR_{GSH}, which represents the absolute amount of GSH that is produced per unit of time [mg/(kg · d)], can be calculated using the following equation:

$$ASR_{GSH}(\text{mg/(kg·d)} = FSR_{GSH}/(100 \times \text{conc.} \times 307 \times \text{ht} \times 0.075)$$

where conc. is GSH concentration in mmol/L packed erythrocytes, 307 is the molecular weight of GSH, ht is hematocrit, and 0.075 is the estimated circulating volume in a preterm neonate, expressed as L/kg.

Amino acid concentrations. Plasma concentrations of direct GSH precursors glutamate, glycine, and cysteine (measured as cystine), and indirect precursors glutamine, methionine, and serine, were determined with a Biochrom 30 amino acid analyzer, using ninhydrin detection (Biochrom Ltd., Cambridge, England).

Plasma markers of oxidative stress. We measured AOPP in plasma by the spectrophotometric assay described by Witko-Sarsat *et al.* (14). The AOPP were calibrated with chloramine-T solutions that absorb at 340 nm in the presence of potassium iodide. Because the absorbance of chloramine-T at 340 nm is linear up to 100 μM, AOPP concentrations were expressed as μmol/L chloramine-T equivalents.

Statistics. Statistical analyses were performed using SPSS v14.0 (SPSS Inc., Chicago, IL) and Graph Prism v4 (GraphPad Software Inc., San Diego, CA). Data are expressed as means ± SD or as medians (min–max). Primary outcome of this study was the glutathione fractional synthesis rate. Power calculation shows that, to detect a difference in FSR_{GSH} of 10%/d and a SD of 6% [based on our earlier study (13)] with an α of 0.05 and a power of 0.80, group size needed to eight. We included eight and 10 infants in each group. Differences between groups were determined using independent *t* tests or Mann-Whitney tests in case of normal or skewed distribution of the population, respectively. For differences in frequency of mode of delivery, χ^2 test was used. A *p* value <0.05 was considered as statistically significant.

RESULTS

The stable isotope infusion was started either on day 1 (7 ± 4.8 h after birth, early group) or day 2 (31 ± 5.9 h after birth, late group). Clinical characteristics are displayed in Table 1. The maximum inspired oxygen fraction was significantly higher in the group of infants measured on day 2. Nutritional intakes before and during the study are shown in Table 2.

Concentrations of plasma precursor AAs. Table 3 shows the plasma concentrations of all AAs involved in GSH synthesis with reference values obtained from healthy term breast-fed infants (19). Cystine concentration in the late group was significantly lower than in the early group and was below the reference value (19). Concentrations of glutamate, glutamine, methionine, and serine were not different between the groups, but glutamate concentrations were below reference values in both groups.

Table 1. *Clinical characteristics*

	Early group	Late group
N (male:female)	8 (7:1)	10 (8:2)
Gestational age (wk)	$28^2/_7 \pm {}^4/_7$	$27^4/_7 \pm 2^0/_7$
Birth weight (g)	1023 ± 180	961 ± 288
Birth weight *Z*-score	−0.8 ± 1.1	−1.0 ± 1.6
Mode of delivery (vaginal:cesarean section)	0:8	5:5*
Apgar score	9 (8–10)	9 (4–9)
CRIB score†	2 (1–4)	4 (1–10)
Cord blood pH	7.28 ± 0.07	7.27 ± 0.17
Cord blood BE (mmol/L)	−5.1 (−6.6 to −1)	−2.5 (−22 to 1.6)
FiO_2		
Minimum (%) on day 1	21 (21–25)	21 (21–21)
Maximum (%) on day 1	30 (21–45)	55 (29–100)*
FiO_2	21 (21–21)	21 (21–21)
Minimum (%) on day 1 + 2		
Maximum (%) on day 1 + 2	31 (21–80)	58 (29–100)*

Values represent either mean ± SD or median (min–max). Mode of delivery and maximum FiO_2 were significantly different between groups. Other characteristics were not different between groups.

* $p < 0.05$.

† The CRIB score (Clinical Risk Index for Babies) indicates the degree of illness (17). The score has a maximum of 23 points and is positively correlated with the severity of illness.

Table 2. *Nutritional intakes on each study day*

	Day 1	Day 2
Nonprotein energy intake, kcal/(kg · d)	30 ± 4	40 ± 12*
Amino acid intake, g/(kg · d)	2.3 ± 0.5	2.4 ± 0.1

Values are expressed as mean ± SD (*t* test). As anticipated, infants received increased caloric intake on day 2 (late group) compared with day 1 (early group).

* $p < 0.05$.

Table 3. *Plasma AA concentrations in μmol/L*

	Term healthy infants*	Preterm early group ($n = 8$)	Preterm late group ($n = 9$)
Methionine	21–50	46 ± 17	51 ± 19
Cystine	33–55	51 ± 11	31 ± 11†
Glutamate	76–551	66 ± 27	77 ± 46
Glutamine	147–623	657 ± 270	636 ± 114
Glycine	66–432	386 ± 166	344 ± 54
Serine	79–227	214 ± 94	194 ± 53

Values are expressed as mean ± SD (*t* test). AA concentrations for one patient of the day 2 group could not be determined because of shortage of plasma.

* Reference levels of plasma amino acid concentrations in healthy term breast-fed infants (19).

† $p < 0.002$.

GSH concentrations and synthesis rates. Free intraerythrocytic [1-^{13}C]glycine enrichments reached a plateau after 4 h of infusion. Enrichments did not differ between the groups (mean 3.9 ± 0.9 and 3.4 ± 0.5 MPE in the early and late group, respectively, $p = 0.18$).

GSH kinetic data are shown in Figure 1. The concentration of erythrocyte GSH was significantly higher in the late group. However, FSR_{GSH} was not different between groups. In addition, the ASR_{GSH} did not differ significantly between the groups ($p = 0.054$).

Plasma levels of AOPP. Plasma AOPP levels are shown in Figure 2. No differences were found between groups for plasma concentration of AOPP. The mean plasma concentration of AOPP of both groups was 217 ± 108 μM, which is in agreement with earlier studies on AOPP levels in preterm infants and indicates the presence of oxidative stress compared with term infants (15,20,21).

DISCUSSION

In preterm infants, there is a rapid decline of GSH immediately after birth, possibly as a result of higher oxidative stress after birth. Recently, we demonstrated that early AA administration directly after birth leads to an increased GSH concentration on postnatal day 2 (13). In this study, preterm infants receiving AAs from birth onward showed an age-related rise in GSH concentration without a concomitant rise in GSH synthesis rate, despite a higher inspired oxygen fraction in the late group.

The ASR_{GSH} tended to be higher in the infants studied on day 2 ($p = 0.054$) with a difference of 2.5 mg/(kg · d) but was not significant between groups. Power calculation based on ASR_{GSH} showed that at least four infants in each group should have been included to reach a physiologic difference of 4.5 mg/(kg · d). In this study, eight and 10 infants were included and therefore, this study has enough power. When speculating on a potential difference in ASR with an equal FSR, the observed difference in ASR would probably be almost exclusively attributed to the difference in concentration.

Because no differences were found in GSH synthesis rates, it would seem that the increased GSH concentration accompanying early AA administration is not caused by increased synthesis, suggesting that GSH consumption decreases upon AA administration.

Intracellular GSH concentration is determined by *de novo* synthesis; recycling of GSSG (oxidized, dimeric form of GSH) back to GSH; transport to the extracellular space; and utilization by peroxides, transferases, transhydrogenases, and transpeptidases (22). The method for measuring GSH concentration in this study does not discriminate between reduced and oxidized GSH. Therefore, the increased total GSH concentration in the late group cannot be explained by different recycling rates between the groups. Moreover, recycling of oxidized GSSG back to GSH seems to be enhanced rather than decreased in preterm infants (23,24). Consequently, the increased GSH concentration is likely to be caused by decreased consumption of GSH after early AA administration in preterm infants.

There are several possible explanations for this decreased GSH consumption upon AA administration. First, AAs can serve as antioxidants themselves. A study in healthy elderly people demonstrated reduced oxidative stress after the administration of essential AAs, including methionine and cysteine (both sulfur AAs) (25). It is possible that the latter two AAs were responsible for the antioxidant effect. Previous studies indeed showed that methionine residues may protect proteins from critical oxidative damage (26), and that oral supplements with whey proteins, which contain high amounts of sulfur AAs, increase plasma GSH levels in patients with HIV (27). The mechanism behind this increase in plasma GSH remains to be elucidated, because kinetic studies were not performed. Another possible explanation is that increased availability of AAs, as shown by increased plasma concentrations (13), up-regulates synthesis of other antioxidants. van den Akker *et al.* (28) showed that AA administration to preterm infants results in increased albumin synthesis and albumin also exerts antioxidant properties (29). Increased levels of other antioxidants might decrease the consumption of GSH, resulting in its increased concentrations and availability.

Besides antioxidant properties, GSH also functions as a cysteine reservoir. Consequently, GSH is broken down in response to shortage of cysteine and compromised protein synthesis. This follows from the observation that GSH levels become depleted if intakes of sulfur amino acids are minimal but sufficient to maintain protein synthesis at adequate levels (30,31). It is therefore possible that increased availability of cysteine reduces breakdown of GSH to generate free cysteine. Besides increased GSH concentration, plasma cysteine concentrations were also increased in infants receiving AAs from birth onward (13), suggesting decreased necessity for GSH breakdown to release cysteine. In this study, however, plasma cysteine (measured as cystine) concentrations dropped in the first day after birth resulting in lower plasma cysteine concen-

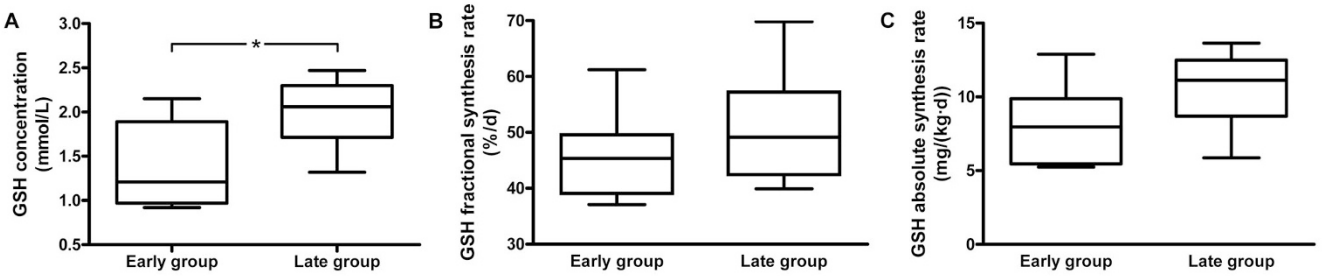

Figure 1. Glutathione concentrations (*A*), fractional synthesis rates (*B*), and absolute synthesis rates (*C*) in erythrocytes in the early ($n = 8$) and late group ($n = 10$) expressed as box plots. Glutathione concentrations were significantly higher in the late group compared with the early group ($p < 0.05$, *t* test). There were no significant differences in fractional synthesis rates and absolute synthesis rates between groups (*t* test).

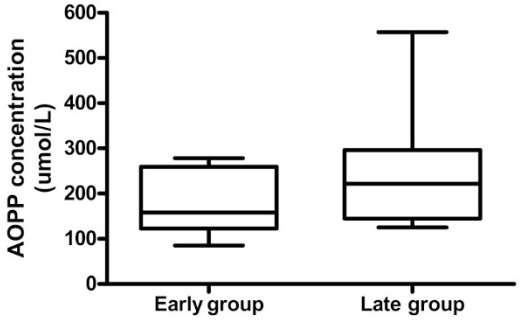

Figure 2. Plasma concentrations of advanced AOPP (expressed as μmol/L chloramine-T equivalents) in preterm infants early ($n = 8$) and late ($n = 10$) after birth. Data are expressed as box plots. There were no statistically significant differences in AOPP concentrations between groups (*t* test).

trations in the late group, whereas erythrocytic glutathione concentrations had increased. Although plasma and erythrocytes represent two compartments, this decrease suggests that serving as a substrate for GSH synthesis is the metabolic fate of cysteine, most likely due to increased requirements imposed by extrauterine life. However, it is recently demonstrated that a further increase in cyteine intake did not result in an increased GSH synthesis (32). Alternatively, cysteine might be used for synthesis of important proteins such as albumin.

AOPP concentrations typically increase significantly in the first week in preterm infants, both in hypoxic and nonhypoxic infants (20). In this study, however, plasma AOPP concentrations were not yet increased in the late group, even though the maximum inspired oxygen fraction was significantly higher in this group. It might well be that antioxidant defense mechanisms, such as the increased availability of GSH on day 2, avert some of the oxidative stress in preterm infants after birth.

There are some limitations in this study. First, it would have been useful to quantify oxidative stress in a broader perspective, going beyond protein oxidation only. For example, determining isoprostanes as a marker for lipid peroxidation (33). Furthermore, the ratio between erythrocytic reduced and oxidized GSH is also suggested to be an excellent marker for oxidative stress and is already used to this aim in infants (34). Regrettably, we could not obtain enough plasma for determining these markers, as a consequence of limited possibilities of blood withdrawal in these preterm infants. Second, the proportion of cesarean sections in the early group was significantly higher than that in the late group. It is argued that the mode of delivery might influence the degree of oxidative stress in the neonate, but literature on this topic is contradictory. Some authors report increased oxidative stress, whereas others report diminished oxidative stress (35–38).

At first glance, it can be said that the ranges of the FSR appear wide. The ranges are not caused by outliers but are caused by variation of glutathione FSR within the population. Although the range of fractional synthesis rates is between 30 and 70%/d, the SD is low in both groups compared with similar studies conducted by others (8,39,40).

In this study, we demonstrate an increase of GSH concentration in the first days after birth in preterm infants receiving AAs, without a concomitant rise in GSH synthesis. Normally, glutathione is rapidly depleted after birth in preterm infants, possibly as a consequence of increased oxidative stress postnatally. Whether AA could serve as antioxidants themselves, promote synthesis of other antioxidants, and thereby reduce the need for glutathione as an antioxidant, or whether the increased plasma cystine concentrations (13) decrease GSH breakdown to release free cysteine remains to be clarified.

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