

Evidence for Accelerated Rates of Glutathione Utilization and Glutathione Depletion in Adolescents With Poorly Controlled Type 1 Diabetes

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Depletion of glutathione, an important antioxidant present in red cells, has been reported in type 1 diabetes, but the mechanism of this depletion has not been fully characterized. Glutathione depletion can occur through decreased synthesis, increased utilization, or a combination of both. To address this issue, 5-h infusions of L-[3,3-²H₂]cysteine were performed in 16 diabetic adolescents divided into a well-controlled and a poorly controlled group and in eight healthy nondiabetic teenagers as control subjects (HbA_{1c} 6.3 ± 0.2, 10.5 ± 0.6, and 4.8 ± 0.1%, respectively). Glutathione fractional synthesis rate was determined from ²H₂-cysteine incorporation into blood glutathione. We observed that 1) erythrocyte cysteine concentration was 41% lower in poorly controlled patients compared with well-controlled patients (*P* = 0.009); 2) erythrocyte glutathione concentration was ~29% and ~36% lower in well-controlled and poorly controlled patients compared with healthy volunteers; and 3) the fractional synthesis rate of glutathione, although similar in well-controlled and healthy subjects (83 ± 14 vs. 82 ± 11% per day), was substantially higher in the poorly controlled group (141 ± 23% per day, *P* = 0.038). These findings suggest that in diabetic adolescents, poor control is associated with a significant depletion of blood glutathione and cysteine, due to increased rates of glutathione utilization. This weakened antioxidant defense may play a role in the pathogenesis of diabetes complications. *Diabetes* 54:190–196, 2005

Glutathione (γ-glutamyl-cysteinyl-glycine), the most abundant small peptide in the body, is present at millimolar concentrations in the intracellular milieu in most tissues and plays a pivotal role in the defense against oxidative stress (1). In human blood, the bulk of glutathione is found inside erythrocytes (~2 mmol/l), whereas the plasma glutathione

concentration is extremely low (~10 μmol/l). In erythrocytes, glutathione is the main “redox buffer” in charge of the maintenance of iron in its reduced form, a prerequisite for hemoglobin’s function. Erythrocyte glutathione has a rapid turnover rate (~60–100% per day) (2–6) and arises from de novo synthesis from its constituent amino acids glutamate, cysteine, and glycine.

Depletion of blood glutathione has been documented in many clinical situations such as malnutrition (3), severe burn injury (4), human immunodeficiency virus (HIV) infection (5), and diabetes, both type 1 and type 2 (7–9). Glutathione depletion may have adverse consequences in diabetic patients, independent of glycemic control, and it may weaken the defense against oxidative stress. This could cause damage to protein, DNA, or membrane lipids and thus potentially lead to cell dysfunction in various tissues. In diabetes, increased oxidative stress is known to play a decisive role in the pathogenesis of vascular complications (10,11). In addition, glutathione depletion may adversely affect insulin sensitivity, since oxidative stress impairs insulin-sensitive glucose transport in adipocytes (12), potentially through activation of nuclear factor κB (13). In contrast, glutathione infusion was found to enhance glucose disposal in patients with type 2 diabetes (14) and decreased erythrocyte sorbitol levels (15). The latter is a well-known factor in the development of diabetic neuropathy and eye disease. Finally, experimental studies suggest oxidative stress alters insulin secretion by damaging islet β-cells (16), and systemic measures of oxidative stress correlate with insulin requirement in patients with type 1 diabetes (17).

Although glutathione depletion has been observed in diabetes, the mechanisms responsible for this depletion are not fully understood. Because the glutathione pool is turning over rapidly, depletion of blood glutathione could result from 1) a decreased rate of synthesis, 2) an increased rate of utilization, or 3) a combination of the two. Recently, we developed a method to determine the rate of glutathione synthesis in vivo, by monitoring the incorporation of a labeled precursor amino acid into newly formed blood glutathione during the course of a stable isotope-labeled amino acid infusion (18). The aim of the present study was therefore to determine 1) whether the rate of blood glutathione synthesis was altered in adolescents with uncomplicated type 1 diabetes and, 2) if so, whether these alterations were related to the degree of glucose control. The kinetics of erythrocyte glutathione were thus

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FSR, fractional synthesis rate; MPE, mole percent excess.

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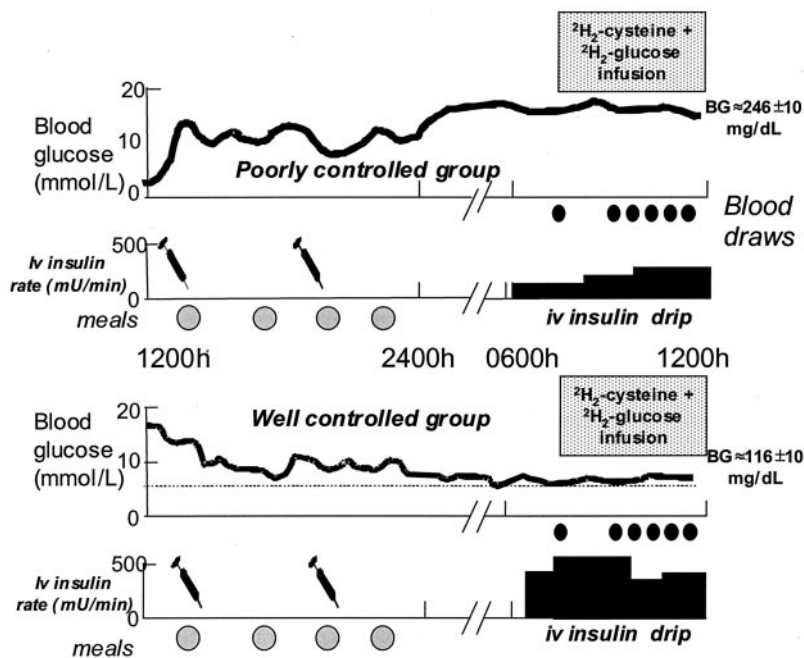


FIG. 1. Protocol for the determination of cysteine and glutathione kinetics in adolescents with type 1 diabetes. A similar protocol (without any insulin infusion) was carried out in the nondiabetic control subjects. BG, blood glucose; iv, intravenous.

assessed using infusions of stable isotope-labeled cysteine in three groups of matched adolescents: 1) healthy control volunteers, 2) well-controlled diabetic subjects, and 3) poorly controlled diabetic teenagers.

RESEARCH DESIGN AND METHODS

The day before each infusion study, solutions of sterile, pyrogen-free D-[6,6- $^2\text{H}_2$]glucose and L-[3,3- $^2\text{H}_2$]cysteine (98 and 97.5% $^2\text{H}_2$, respectively; both purchased from Cambridge Isotope Laboratories, Andover, MA) were prepared using an aseptic technique by dissolving accurately weighed amounts of tracers in known volumes of sterile, 0.9% saline, passed through a 0.22- μm Millipore filter and stored at 4°C in sterile sealed vials until infusion. Homogluthathione (glutamyl-cysteinyl-alanine) was obtained from Bachem Chemicals (King of Prussia, PA), and all other chemicals came from Sigma-Aldrich (St. Louis, MO).

A total of 24 adolescents participated in the study, after written informed consent was obtained from themselves and their parents or legal guardians, according to protocols approved by the Nemours Children's Clinic Research Committee and the Institutional Review Committee at Baptist Medical Center (Jacksonville, FL). Patients were enrolled in three different groups of eight adolescents each: 1) a group with poorly controlled type 1 diabetes, as defined by $\text{HbA}_{1c} > 9\%$ after > 6 months of diabetes, with no evidence of renal, ocular, or vascular complication; 2) a group with well-controlled type 1 diabetes, as defined by $\text{HbA}_{1c} < 7\%$ after > 6 months of diabetes, and with no evidence of diabetes complications; and 3) healthy matched nondiabetic adolescents who served as control subjects.

Protocol design is depicted in Fig. 1. On the night before the isotope infusion study, each subject was admitted to the Clinical Research Center at Wolfson Children's Hospital, Jacksonville, Florida. Weight was measured, and body fat mass was assessed by determination of skinfold thickness with Harpenden calipers. Each diabetic subject received his or her usual dose of short-acting insulin by subcutaneous injection before dinner. Those wearing an insulin pump were kept on their usual boluses and basal subcutaneous insulin infusion rates overnight, and the patients who were using multiple shots were kept on their usual nighttime insulin regimen. All remained fasting overnight until completion of the study at 1300 the following day.

On the following morning at 0700, two short catheters were placed: one in a forearm vein for isotope infusion and the other in a contralateral hand vein. During the sampling period, the hand was placed in a heating pad at 60°C to obtain arterialized venous blood (19). During the morning of study, patients remained fasting and did not receive any subcutaneous insulin, but each patient was connected to an intravenous drip of regular human insulin. Blood glucose was monitored at 30-min intervals, and the rate of intravenous insulin was adjusted so as to maintain blood glucose either between 80 and 160 mg/dl (for the "well-controlled" group) or between 180 and 240 mg/dl for the "poorly controlled" group, until completion of the isotope infusion at 1300. Serum

electrolytes and urine ketones were checked periodically throughout the infusion.

At 0800, a primed continuous infusion of L-[3,3- $^2\text{H}_2$]cysteine and D-[6,6- $^2\text{H}_2$]glucose (5 and 20 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, respectively) was started and continued for 5 h. A total of 10 ml arterialized venous blood was obtained at -15 and 0 min and at 30-min intervals between 120 and 300 min of the isotope infusion, for measurement of hematocrit, plasma glucose concentration, and $^2\text{H}_2$ -glucose enrichment and for the determination of the concentration and $^2\text{H}_2$ enrichment of erythrocyte free cysteine and glutathione. At 1300, isotope infusion was discontinued, and patients received an appropriate dose of subcutaneous regular insulin, ate lunch, and were discharged from the hospital. The healthy subjects underwent similar isotope infusion studies, but without any insulin administered.

Analytical procedures. Erythrocyte free cysteine was analyzed using a modification of the method described by Capitan et al. (20). Blood was collected into ice-cold EDTA glass tubes and immediately centrifuged at 5,000g for 20 min at 4°C to separate the red cell pellet from plasma. Plasma was discarded and replaced by an equal volume of ice-cold distilled water to hemolyze red cells. Samples were vortexed and stored at -80°C until analysis. A 1.5-ml aliquot of hemolyzed red cell sample was pipetted into a glass tube containing 850 μl of 0.2 mol/l phosphate buffer, 39 μmol dithiothreitol, and 1.5 μmol penicillamine (dimethylcysteine) as an internal standard. Samples were shaken, adjusted to pH 8.5 with 0.8 mol/l NaOH, and left at room temperature for 15 min. Samples were then deproteinized with 375 μl 50% sulfosalicylic acid, spiked with 375 μl of 0.2 mol/l phosphate buffer, vortexed until homogenization, and then centrifuged at 3,000g for 15 min at 10°C. Supernatants were collected and adjusted to pH 7.5 with 0.8 mol/l NaOH. After adding 200 μl ethylchloroformate (to derivatize cysteine's NH_2 and SH groups), samples were shaken for 10 min at room temperature, then adjusted to pH 1.5 using 1 mol/l HCl, and transferred into screw-cap vials containing 0.5 g NaCl. Cysteine was extracted by shaking for 1 min in 4 ml peroxide-free diethyl ether. The ethereal phase was collected and evaporated under nitrogen at 50°C, and samples were incubated for 10 min at 80°C with 250 μl of 1 mol/l HCl in methanol to methylate cysteine's carboxylic group. Samples were evaporated under nitrogen at room temperature, and the dry residue was dissolved in 400 μl ethylacetate and analyzed using an HP 5971 gas chromatograph mass spectrometer as described (20). Ions at $m/z = 220$ and 222, representing natural and $^2\text{H}_2$ -cysteine, respectively, were selectively monitored to assess $^2\text{H}_2$ -cysteine enrichments; ions at $m/z = 160$ and 147, representing natural cysteine and penicillamine, respectively, were used to assess cysteine concentrations. Erythrocyte cysteine concentrations are expressed per milliliter of whole blood and represent both free cysteine and free cysteine concentrations.

The concentration and isotopic enrichment of erythrocyte glutathione were determined by gas chromatography mass spectrometry as described (18), using homogluthathione as an internal standard. Plasma $^2\text{H}_2$ -glucose

TABLE 1
Selected characteristics of the population studied

	Healthy subjects	Well-controlled type 1 diabetic subjects	Poorly controlled type 1 diabetic subjects
Age (years)	16.8 ± 0.5	15.5 ± 0.3	15.8 ± 0.5
Sex (M/F)	5/3	5/3	5/3
Height (cm)	173 ± 3	175 ± 3	163 ± 7
Weight (kg)	69 ± 5	70 ± 5	67 ± 5
BMI (kg/m ²)	22.9 ± 1.1	22.9 ± 1.1	25.7 ± 2.1
Lean body mass (kg)	49 ± 4	54 ± 4	48 ± 3
Type 1 diabetes duration (years)	0	6 ± 2	6 ± 1
HbA _{1c} (%)	4.8 ± 0.1	6.3 ± 0.2†	10.5 ± 0.6‡‡
Hematocrit (%)	43.1 ± 1.5	42.2 ± 1.1	42.0 ± 1.5
Blood glucose (mg/dl)*	89 ± 2	116 ± 9†	246 ± 10‡‡

Data are means ± SE for eight subjects per group. *Mean blood glucose concentration during the isotope infusion study. †*P* < 0.0001 vs. healthy subjects; ‡‡*P* < 0.0001 vs. well-controlled patients.

enrichments were determined as described previously (21). Plasma glucose concentrations were determined by the glucose oxidase method.

Calculations. Glucose rate of appearance (Ra_{glucose} , $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) was calculated as follows: $Ra_{\text{glucose}} = i_{\text{glucose}} \times [(Ei_{\text{glucose}}/Ep_{\text{glucose}}) - 1]$, where i_{glucose} is the rate of ²H₂-glucose infusion ($\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) and Ei_{glucose} and Ep_{glucose} (mole percent excess [MPE]) are the ²H₂-glucose enrichments in the infusate and in plasma at plateau, respectively. Cysteine rate of appearance (Ra_{cys} , $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) was calculated as follows: $Ra_{\text{cys}} = i_{\text{cys}} \times [(Ei_{\text{cys}}/Erb_{\text{cys}}) - 1]$, where i_{cys} is the rate of ²H₂-cysteine infusion ($\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) and Ei_{cys} and Erb_{cys} (MPE) are the ²H₂ enrichments in the infusate and erythrocyte free cysteine at plateau, respectively. Cysteine metabolic clearance rate ($1 \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) was calculated by dividing the cysteine appearance rate by red cell cysteine concentration.

Glutathione fractional synthesis rate (FSR) (%/day) was calculated as follows: $\text{FSR} = 100 \times 24 \times (\Delta E_{\text{GSH}}/\Delta t)/Erb_{\text{cys}}$, where $\Delta E_{\text{GSH}}/\Delta t$ is the slope (MPE/h) of the regression line describing the rise in erythrocyte ²H₂-glutathione enrichment (MPE) as a function of time (hours) over the last 2 h of isotope infusion, and 24 and 100 convert FSR to percent per day. Glutathione absolute synthesis rate ($\text{mmol} \cdot \text{l}^{-1} \cdot \text{day}^{-1}$) was calculated as follows: $\text{FSR} \times [\text{GSH}]$, where [GSH] is erythrocyte glutathione concentration (mmol/l).

Statistical analysis. Results are expressed as means ± SE. Parameters were compared between treatments using repeated-measures ANOVA and paired *t* tests. Significance was established at *P* < 0.05.

RESULTS

As shown in Table 1, groups were well matched for age, sex, and BMI. By design, the two diabetic groups differed by their glycohemoglobin levels; yet the duration of diabetes did not differ, and none of the diabetic patients had any detectable complications (data not shown).

Glucose kinetics. Blood glucose was maintained at near steady state in all groups, with mean coefficients of variation (coefficient of variation = $100 \times \text{SD}/\text{mean}$) over the last 2 h of the study at 2.9 ± 0.4 , 6.6 ± 2.0 , and $6.1 \pm 0.9\%$ in healthy subjects and well-controlled and poorly controlled diabetic patients, respectively. Mean blood glucose during the last 5 h of the study was 89 ± 2 , 116 ± 9 , and 246 ± 10 mg/dl in healthy volunteers and well-controlled and poorly controlled diabetic subjects, respectively. None of the patients developed ketosis during the study, and serum bicarbonate did not differ between groups (25 ± 2 , 25 ± 2 , and 27 ± 2 mmol/l, NS, in healthy subjects and well-controlled and poorly controlled diabetic patients, respectively). Glucose production (Ra) was 1.924 ± 0.049 , 2.368 ± 0.152 , and 3.084 ± 0.244 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ in healthy adolescents and well-controlled and poorly controlled adolescents, respectively. As expected, Ra values were higher in both diabetic groups (*P* = 0.012 and *P* = 0.0002 vs. control subjects) than in control

subjects, and the difference between the diabetic groups was significant as well (*P* = 0.039).

Cysteine kinetics. Erythrocyte cysteine concentrations remained constant throughout isotope infusion. Erythrocyte cysteine concentration was slightly, but not significantly (*P* = 0.139), higher in well-controlled diabetic patients (30 ± 3 $\mu\text{mol/l}$) compared with healthy volunteers (23 ± 3 $\mu\text{mol/l}$). In contrast, it was 41% lower (*P* = 0.009) in poorly controlled diabetic subjects (18 ± 2 $\mu\text{mol/l}$) compared with the well-controlled group of diabetic patients (30 ± 3 $\mu\text{mol/l}$). The difference between the poorly controlled group and healthy subjects did not reach statistical significance (18 ± 2 vs. 23 ± 3 $\mu\text{mol/l}$, *P* = 0.189) (Fig. 2). Cysteine appearance rate did not differ between groups (69 ± 8 , 68 ± 6 , and 65 ± 5 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ in

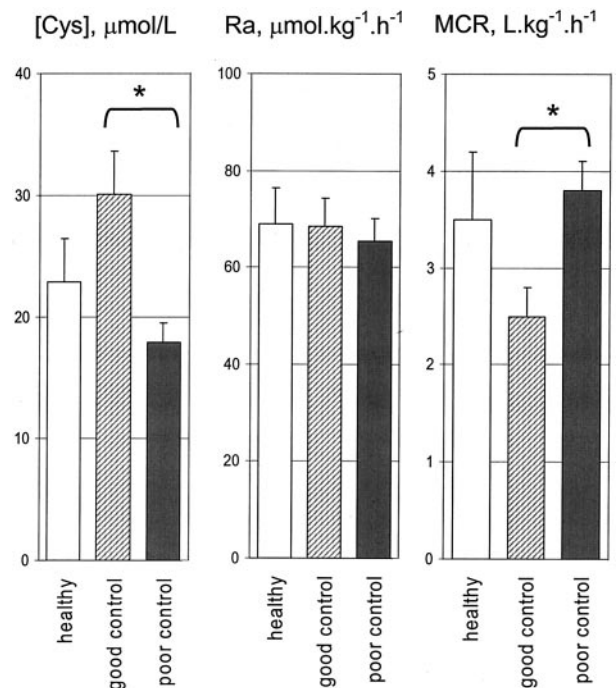


FIG. 2. Erythrocyte free cysteine concentration ([Cys]), cysteine rate of appearance (Ra) (as estimated from erythrocyte ²H₂-cysteine enrichments), and metabolic clearance rate (MCR) (MCR = $Ra/[Cys]$) in adolescents with well-controlled and poorly controlled type 1 diabetes compared with matched healthy adolescents. **P* < 0.05.

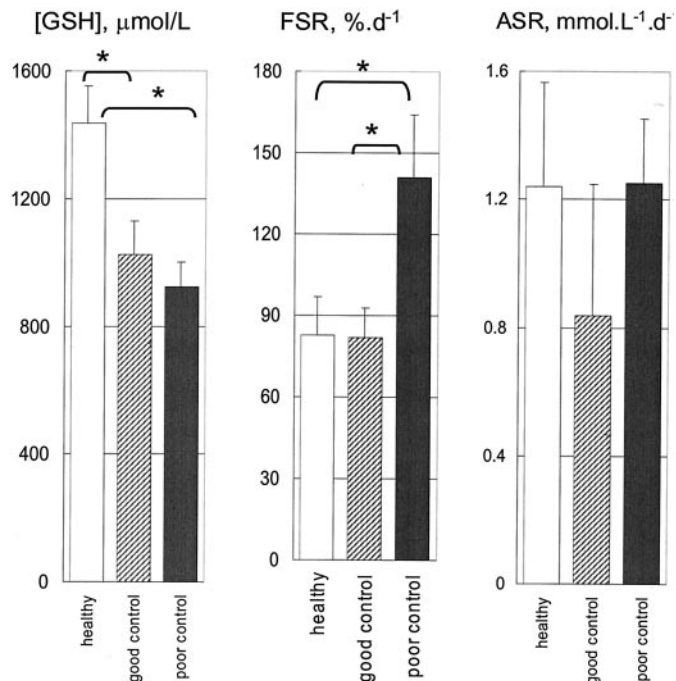


FIG. 3. Erythrocyte glutathione concentration (GSH), FSR, and absolute synthesis rate (ASR) in adolescents with well-controlled and poorly controlled type 1 diabetes compared with matched healthy adolescents. * $P < 0.05$.

healthy volunteers and well-controlled and poorly controlled patients, respectively). As a consequence of the lower cysteine concentration, cysteine metabolic clearance rate was significantly elevated in the poorly controlled diabetic subjects compared with well-controlled diabetic subjects (3.8 ± 0.3 vs. $2.5 \pm 0.3 \text{ l} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; poor control versus good control, $P = 0.013$) but not when compared with healthy subjects (3.8 ± 0.3 vs. $3.5 \pm 0.7 \text{ l} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; $P = 0.686$).

Glutathione kinetics. Erythrocyte glutathione concentration (Fig. 3) was $\sim 29\%$ lower in the well-controlled diabetic group compared with the healthy volunteers ($1,435 \pm 120$ vs. $1,026 \pm 103 \text{ } \mu\text{mol/L}$, $P = 0.002$) and $\sim 36\%$ lower in the poorly controlled group ($1,435 \pm 120$ vs. $922 \pm 77 \text{ } \mu\text{mol/L}$, healthy subjects versus poorly controlled patients, $P = 0.015$) and correlated negatively with HbA_{1c} (Fig. 4). The difference between the two diabetic groups was not statistically significant ($P = 0.405$).

Because glutathione synthesis occurs inside red cells, precursor $^2\text{H}_2$ -cysteine enrichment was measured in erythrocytes and reached steady state in all groups by the end of the third hour of isotope infusion, whereas the $^2\text{H}_2$ enrichment in erythrocyte glutathione increased linearly with time (Fig. 5). The FSR of erythrocyte glutathione, calculated from the rate of incorporation of labeled cysteine into blood glutathione as a function of time, was $83 \pm 14\%/ \text{day}$ in healthy adolescents (Fig. 3). Whereas glutathione FSR was unaltered in well-controlled diabetic subjects ($82 \pm 11\%/ \text{day}$, $P = 0.964$), it was substantially ($\sim 70\%$) higher in poorly controlled diabetic patients, when compared with either healthy subjects (141 ± 23 vs. $83 \pm 14\%/ \text{day}$; $P = 0.038$) or the well-controlled group (141 ± 23 vs. $82 \pm 10\%/ \text{day}$; $P = 0.028$). Glutathione absolute synthesis rate, as calculated by $\text{FSR} \times \text{glutathione concentration}$,

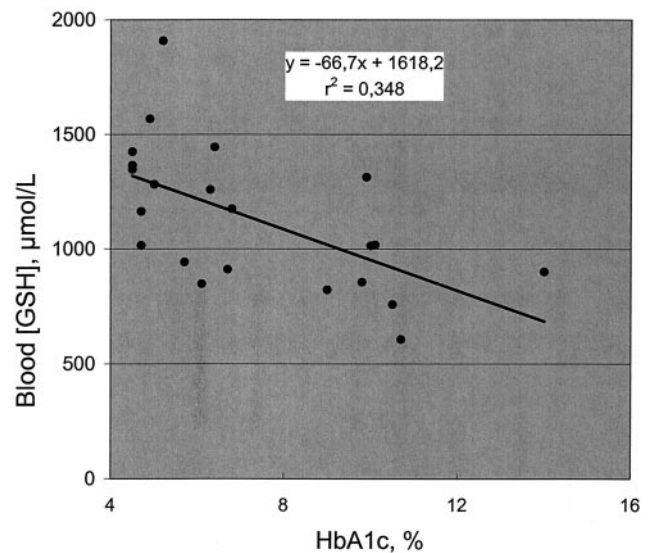


FIG. 4. Correlation between blood glutathione concentration and HbA_{1c} in the 24 adolescents studied.

did not differ between groups (1.239 ± 0.325 , 0.835 ± 0.135 , and $1.252 \pm 0.179 \text{ mmol} \cdot \text{l}^{-1} \cdot \text{day}^{-1}$ in control subjects and well-controlled and poorly controlled patients, respectively).

DISCUSSION

The present study demonstrates that 1) blood glutathione is significantly depleted in adolescents with uncomplicated type 1 diabetes, compared with an age- and sex-matched group of healthy volunteers; and 2) the depletion is not due to a decrease in the rate of glutathione synthesis, but occurs despite either unaltered or accelerated rates of glutathione fractional synthesis, depending on the quality of blood glucose control. The results further provide evidence for a depletion of blood cysteine in poorly controlled diabetic adolescents, potentially due to increased use of cysteine in the synthetic pathway for glutathione. Taken in aggregate, the data suggest glutathione depletion arises from increased glutathione utilization. This is particularly compelling, considering these are young subjects in adolescence. Regardless of the mechanism(s) involved, depletion of glutathione and cysteine may have deleterious long-term consequences, since it may impair the body's antioxidant capabilities, and oxidative stress is presumed to play a key role in the pathophysiology of diabetes complications.

Cysteine is considered a semi-essential amino acid, as it can be synthesized from methionine, an essential sulfur amino acid. Yet cysteine has been found to be the limiting amino acid for glutathione synthesis in patients suffering from malnutrition (22), HIV infection (2), or severe burns (5). We are not aware of previous studies of cysteine turnover in patients with type 1 diabetes. Although erythrocyte cysteine concentration and turnover were found to be normal in the group of adolescents who were in good control, the small number of patients enrolled in the current study may have precluded the detection of more subtle alterations in the well-controlled group. We nevertheless observed significant alterations in cysteine metabolism in the poorly controlled diabetic teenagers. Because

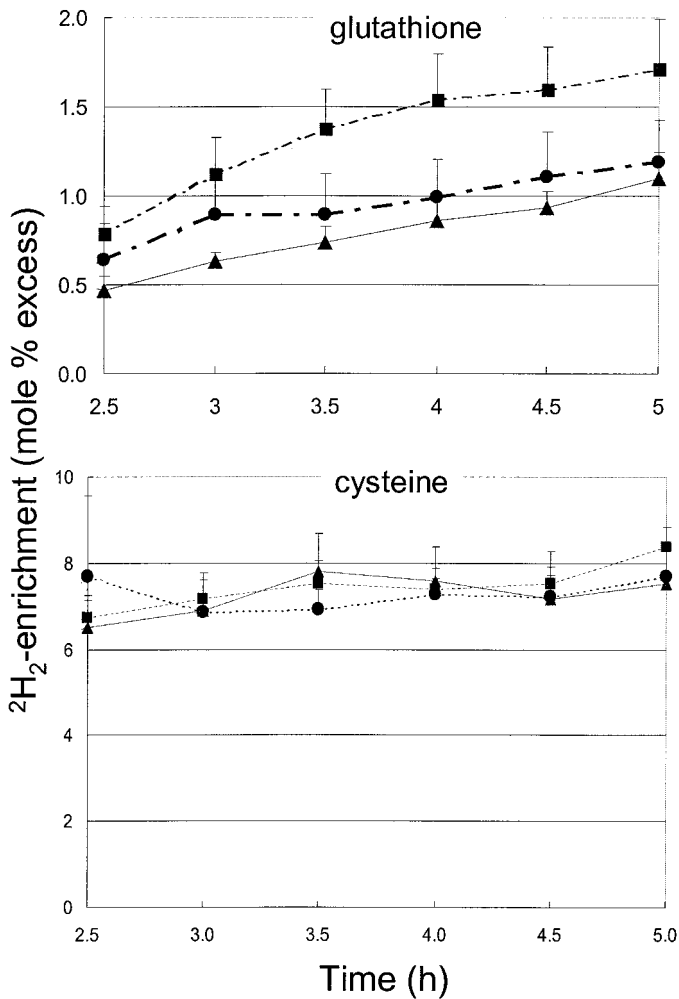


FIG. 5. Time course of $^2\text{H}_2$ enrichment in erythrocyte free cysteine and glutathione during a 5-h infusion of L-[3,3- $^2\text{H}_2$]cysteine in healthy adolescents and adolescents with well-controlled and poorly controlled type 1 diabetes. \blacktriangle , healthy; \bullet , good control; \blacksquare , poor control.

erythrocyte cysteine concentration—expressed in micromoles per milliliter of whole blood in the current report—ultimately represents the amount of cysteine present in a given volume of fluid, alterations in blood volume could, in theory, result in artificially low cysteine levels. Elevation of blood glucose level can alter blood osmolality and, by attracting water into systemic circulation, dilute blood substrates in poorly controlled diabetic patients, which would result in artificially lower cysteine concentrations. Dilution does not however seem to account for the lower cysteine concentrations in the current study, since 1) hematocrit did not differ between groups (Table 1) and, 2) accordingly, when erythrocyte cysteine concentrations were recalculated per milliliter of red blood cells—using a correction for hematocrit—erythrocyte cysteine concentrations (53 ± 19 , 70 ± 10 , and $43 \pm 3 \mu\text{mol/l}$ in healthy subjects and well-controlled and poorly controlled diabetic patients, respectively) were still significantly lower ($P = 0.02$) in poorly controlled diabetic subjects compared with well-controlled diabetic subjects.

In the postabsorptive state, maintenance of the cysteine pool depends on the balance between endogenous cysteine production and cysteine utilization. Overall, cysteine production, as estimated based on erythrocyte-labeled cysteine enrichments, was unaltered in the diabetic patients in the current study. This may seem paradoxical. A higher rate of cysteine release from protein breakdown is

indeed likely in type 1 diabetic patients, since 1) insulin is a potent inhibitor of protein breakdown (23) and 2) insulin deficiency has consistently been found to be associated with a rise in leucine R_a in poorly controlled type 1 diabetes (24,25). Yet, contrary to leucine, cysteine is not an essential amino acid, so cysteine release from proteolysis is not the sole source of endogenous cysteine, and other sources contribute free cysteine in the postabsorptive state, such as 1) de novo cysteine synthesis from methionine and 2) degradation of glutathione to its constituent amino acids, glutamate, cysteine, and glycine. We have every reason to assume that cysteine release from protein breakdown was increased; we therefore speculate that either cysteine de novo synthesis or the release of cysteine from glutathione breakdown was lower in the poorly controlled diabetic group. Because conversion of glutathione to cysteine is a major source of cysteine (26), the depletion in blood glutathione in poorly controlled patients may be responsible for a decrease in cysteine release, which may offset the rise in cysteine release from proteolysis.

The depleted cysteine pool, despite unaltered overall cysteine production, nevertheless points to increased cysteine “wasting” as a likely mechanism for cysteine depletion, as suggested by the increased cysteine metabolic clearance rate in the poorly controlled patients (Fig. 2). Increased urinary loss of cysteine could, in theory, con-

tribute to the lower cysteine concentration. As incorporation of cysteine into glutathione is a major pathway in cysteine utilization (26), increased erythrocyte glutathione synthesis could contribute as well, since glutathione synthesis was enhanced in the poorly controlled group. Accordingly, the absolute synthesis rate of glutathione accounted for $6.4 \pm 0.9\%$ of cysteine turnover in the poorly controlled group compared with $4.2 \pm 0.8\%$ in the well-controlled group ($P = 0.035$, one-tail t test).

The 29–36% depletion in blood glutathione observed in the current study is consistent with the 17–32% lower glutathione concentrations reported by others in patients with both type 1 (7,8) and type 2 diabetes (9), compared with control subjects. Because glutathione has a rapid turnover, maintenance of the glutathione pool depends on its rates of synthesis and utilization. Erythrocyte glutathione arises from de novo glutathione synthesis from its constituent amino acids. Glutathione synthesis begins with the formation of γ -glutamyl-cysteine, a reaction catalyzed by γ -glutamylcysteine synthase, and the subsequent addition of the glycine moiety by glutathione synthetase. Although alteration in any of these steps can obviously alter the cell's capacity to maintain its glutathione pool, the current study rules out impairment of synthesis as a potential mechanism. Depending on the quality of blood glucose control, glutathione fractional synthesis rate indeed was either unaltered or dramatically elevated (in the poorly controlled group). Moreover, glutathione absolute synthesis rate, an index of the overall amount of glutathione produced per day, did not differ significantly between the groups. Although Murakami et al. (27) measured decreased rates of glutamyl-cysteine synthetase in red cells from diabetic patients, enzyme activities measured in isolated cells in vitro may not reflect the exact conditions prevailing in vivo.

If the erythrocyte glutathione pool is depleted despite an unchanged or an increased rate of synthesis, we have to postulate an increased rate of glutathione utilization. Glutathione can be used to "detoxify" reactive oxygen species such as hydrogen peroxide (H_2O_2), a process in which glutathione is oxidized to the dimer glutathione disulfide in a reaction catalyzed by glutathione peroxidase. Reduced glutathione in turn is regenerated from glutathione disulfide by glutathione reductase in a reaction requiring NADPH as a cofactor (28). NADPH is produced solely by the conversion of glucose-6-phosphate to 6-phosphogluconolactone in the pentose phosphate pathway. Because elevation of blood glucose generates oxidative stress through alterations in the mitochondrial electron transport chain (11), increased oxidative stress could contribute to increased glutathione utilization. This is, however, unlikely to happen inside erythrocytes because these cells lack mitochondria. Alternatively, the conversion of glucose to sorbitol in the polyol pathway, a reaction mediated by aldose reductase, uses NADPH as its mandatory cofactor and may compete for NADPH with glutathione reductase, thus impairing glutathione "regeneration." Accordingly, treatment of type 2 diabetic patients with an aldose reductase inhibitor was shown to decrease erythrocyte sorbitol and increase glutathione level (29).

Regardless of the mechanisms responsible for the alterations in blood cysteine and glutathione pools, their de-

pletion may have adverse consequences. In diabetic rodents, changes in erythrocyte glutathione are accompanied by parallel changes in the glutathione content of liver or retina (8,27,30,31), and piglets receiving protein-restricted diets had similar changes in glutathione synthesis rate and concentration in blood and gut mucosa (32). The depletion of blood glutathione observed in the current study therefore may reflect alterations in glutathione pools in other tissues such as vascular endothelium, where impaired antioxidant defense may precipitate diabetes complications (10).

In summary, the current study using stable isotope methodology provides strong evidence for alterations in blood glutathione and cysteine homeostasis in vivo in adolescents suffering from type 1 diabetes when in poor glucose control. The glutathione and cysteine depletion are likely to arise from increased glutathione utilization. It remains to be determined whether the replenishment of blood cysteine and/or glutathione pools can be achieved by dietary measures and whether it would have beneficial effects in adolescents with type 1 diabetes.

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REFERENCES

- West IC: Free radicals and oxidative stress in diabetes. *Diabet Med* 17:171–180, 2000
- Jahoor F, Jackson A, Gazzard B, Pjilips G, Sharpstone D, Frazer ME, Heird W: Erythrocyte glutathione deficiency in symptom-free HIV infection is associated with decreased synthetic rate. *Am J Physiol* 276:E205–E211, 1999
- Reid M, Badaloo A, Forrester T, Morlese JF, Frazer M, Heird WC, Jahoor F: In vivo rates of erythrocyte glutathione synthesis in children with severe protein-energy malnutrition. *Am J Physiol* 278:E405–E412, 2000
- Lyons J, Rauh-Pfeiffer A, Yu YM, Lu XM, Zurakowski D, Tompkins RG, Ajami AM, Young VR, Castillo L: Blood glutathione synthesis rates in healthy adults receiving a sulfur amino acid-free diet. *Proc Natl Acad Sci U S A* 97:5071–5076, 2000
- Yu YM, Ryan CM, Fei ZW, Lu XM, Castillo L, Schultz JT, Tompkins RG, Young VR: Plasma L-5-oxoproline kinetics and whole blood glutathione synthesis rates in severely burned adult humans. *Am J Physiol* 282:E247–E258, 2002
- Lyons J, Rauh-Pfeiffer A, Ming-Yu Y, Zurakowski D, Curley M, Collier S, Duggan C, Nurko S, Thompson J, Ajami A, Borgonha S, Young VR, Castillo L: Cysteine metabolism and whole blood glutathione synthesis in septic pediatric patients. *Crit Care Med* 29:870–877, 2001
- Dominguez C, Ruiz E, Gussinye M, Carrascosa A: Oxidative stress at onset and in early stages of type 1 diabetes in children and adolescents. *Diabetes Care* 21:1736–1742, 1998
- Jain SK, McVie R: Effect of glycemic control, race (white versus black), and duration of diabetes on reduced glutathione content in erythrocytes of diabetic patients. *Metabolism* 43:306–309, 1994
- Elhadd TA, Kennedy G, Hill A, McLaren M, Newton RW, Greene SA, Belch JJ: Abnormal markers of endothelial cell activation and oxidative stress in children, adolescent and young adults with type 1 diabetes with no vascular disease. *Diabetes Metab Res Rev* 15:405–411, 1999

10. Giugliano D, Paolisso G, Ceriello A: Oxidative stress and diabetic vascular complications. *Diabetes Care* 19:257–267, 1996
11. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001
12. Rudich A, Tirosh A, Potashnik R, Hemi R, Kanety H, Bashan N: Prolonged oxidative stress impairs insulin-induced GLUT4 translocation in 3T3-L1 adipocytes. *Diabetes* 47:1562–1569, 1998
13. Ogihara T, Asano T, Katagiri H, Sakooda H, Anai M, Shojima N, Ono H, Fujishiro M, Kushiyama A, Fukushima Y, Kikuchi M, Noguchi N, Aburatani H, Gotoh Y, Komuro I, Fujita T: Oxidative stress induces insulin resistance by activating the nuclear factor-kappa B pathway and disrupting normal subcellular distribution of phosphatidylinositol 3-kinase. *Diabetologia* 47:794–805, 2004
14. Paolisso G, Di Mario G, Pizza G, D'Amore A, Sgambato S, Tesaro P, Varricchio M, D'Onofrio F: Plasma GSH/GSSG affects glucose homeostasis in healthy subjects and non-insulin-dependent diabetics. *Am J Physiol* 263:E435–E440, 1992
15. Ciuchi E, Odetti P, Prando R: The effect of acute glutathione treatment on sorbitol level in erythrocytes from diabetic patients. *Diabetes Metab* 23:58–60, 1997
16. Rabinovitch A, Suarez-Pinzon W, Strynadka K, Lakey JRT, Rajotte RV: Human pancreatic islet β -cell destruction by cytokines involves oxygen free radicals and aldehyde production. *J Clin Endocrinol Metab* 71:152–156, 1996
17. Hoeldtke RD, Bryner KD, McNeill DR, Warehime SS, Van Dyke K, Hobbs G: Oxidative stress and insulin requirements in patients with recent-onset type 1 diabetes. *J Clin Endocrinol Metab* 88:1624–1628, 2003
18. Humbert B, Nguyen P, Obled C, Bobin C, Vaslin A, Sweeten S, Darmaun D: Use of ^{15}N -glutamic acid and homogluthathione to determine both glutathione synthesis and concentration by GCMS. *J Mass Spectrom* 36:726–735, 2001
19. Abumrad NN, Rbain D, Diamond MP, Lacy WW: Use of a heated hand vein as an alternative site for the measurement of amino acid concentrations and for the study of glucose and alanine kinetics in man. *Metabolism* 30:936–940, 1981
20. Capitan P, Malmezat T, Breuillé D, Obled C: Gas chromatographic-mass spectrometric analysis of stable isotopes of cysteine and glutathione in biological samples. *J Chromatogr B Biomed Sci Appl* 732:127–35, 1999
21. Hankard R, Haymond MW, Darmaun D: Role of glutamine as a glucose precursor in fasting humans. *Diabetes* 46:1535–1541, 1997
22. Badaloo A, Reid M, Forrester T, Heird WC, Jahoor F: Cysteine supplementation improves the erythrocyte glutathione synthesis rate in children with severe edematous malnutrition. *Am J Clin Nutr* 76:646–652, 2002
23. Fukagawa NK, Minaker KL, Rowe JW, Goodman MN, Matthews DE, Bier DM, Young VR: Insulin-mediated reduction of whole body protein breakdown: dose-response effects on leucine metabolism in postabsorptive men. *J Clin Invest* 76:2306–2311, 1985
24. Robert JJ, Beaufreere B, Koziat J, Desjeux JF, Bier DM, Young VR, Lestrade H: Whole body de novo amino acid synthesis in type I (insulin-dependent) diabetes studied with stable isotope-labeled leucine, alanine, and glycine. *Diabetes* 34:67–73, 1985
25. Darmaun D, Rongier M, Koziat J, Robert JJ: Glutamine nitrogen kinetics in insulin-dependent diabetic humans. *Am J Physiol* 261:E713–E718, 1991
26. Fukagawa NK, Ajami AM, Young VR: Plasma methionine and cysteine kinetics in response to an intravenous glutathione infusion in humans. *Am J Physiol* 270:E209–E214, 1996
27. Murakami K, Kondo T, Ohtsuka Y, Fujiwara Y, Shimada M, Kawakami Y: Impairment of glutathione metabolism in erythrocytes from patients with diabetes mellitus. *Metabolism* 38:753–758, 1989
28. Hayes JD, McLellan LI: Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defense against oxidative stress. *Free Radic Res* 31:273–300, 1999
29. De Mattia G, Laurenti O, Bravi C, Ghiselli A, Iuliano L, Balsano F: Effect of aldose reductase inhibition on glutathione redox status in erythrocytes of diabetic patients. *Metabolism* 43:965–968, 1994
30. Kern TS, Koluru RA, Engerman RL: Abnormalities of retinal metabolism in diabetes or galactosemia: ATPases and glutathione. *Invest Ophthalmol Vis Sci* 35:2965–2967, 1994
31. Kowluru RA, Engerman RL, Kern TS: Abnormalities of retinal metabolism in diabetes or experimental galactosemia VIII: prevention by aminoguanidine. *Curr Eye Res* 21:814–819, 2000
32. Jahoor F, Wykes L, Reeds PJ, Henry J, Del Rosario M, Frazer M: Protein-deficient pigs cannot maintain reduced glutathione homeostasis when subjected to the stress of inflammation. *J Nutr* 125:1462–1472, 1995