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Acute changes in blood glucose do not alter blood glutathione synthesis in adolescents with poorly controlled type 1 diabetes mellitus

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ARTICLE INFO

Article history:
Received 24 June 2011
Accepted 26 July 2011

ABSTRACT

Depletion of blood glutathione (GSH), a key antioxidant, is associated with type 1 diabetes mellitus (T1D) and contributes to the pathophysiology of diabetes complications. The aim of the current study was to determine whether acute normalization of blood glucose would restore GSH kinetics in adolescents with poorly controlled T1D. Ten 16.9 ± 1.5-year-old (SE) adolescents who had had T1D for 8.5 ± 1.9 years and were free of complications but were in poor control (hemoglobin A_{1c}, 9.2% ± 0.5%) received two 5-hour intravenous infusions of L-[3,3-$^2H_2$]cysteine in the postabsorptive state on 2 separate days after blood glucose had been maintained overnight at 246 ± 24 mg/dL (hyperglycemia) or 118 ± 23 mg/dL (euglycemia) using intravenous insulin infusion. Blood GSH fractional synthesis rates were determined by mass spectrometry from $^2H_2$-cysteine incorporation into GSH. Neither blood GSH (551 ± 169 vs 541 ± 232 μmol/L, $P = .629$) nor GSH fractional synthesis rate (84% ± 30% vs 82% ± 33% d^{-1}, $P = .965$) was altered by the short-term change in glycemic control. This finding suggests that, in adolescents with poorly controlled T1D, either (*a*) blood glucose per se does not regulate GSH metabolism or (*b*) GSH may only respond to sustained, more chronic changes in blood glucose level.

1. Introduction

Glutathione, a tripeptide (γ-glutamyl-cysteinyl-glycine or GSH), is the most abundant antioxidant in the body, particularly in blood where it reaches the millimolar range [1]. Blood GSH is known to be depleted in patients with type 1 (T1D) as well as type 2 diabetes mellitus (T2D) [2-5]. Such depletion is potentially deleterious, as GSH plays a key role in the fight against oxidative stress and oxidative stress contributes to the development of chronic diabetic complications [6,7].

The mechanisms responsible for GSH depletion remain debated. In earlier studies, we observed a significant depletion in blood GSH in adolescents with T1D who were free of any diabetic complication [5], suggesting that the depletion is not a consequence of the complications of diabetes. Furthermore, GSH depletion was due not to a defect in GSH synthesis,

These results were presented, in part, in an abstract form at the meeting of the Pediatric Academic Societies, Baltimore, MD, May 2009 (abstract 3855-62).

Author contributions: DD and NM: designed the study, conducted the studies, overall supervision of studies, analyzed data, and wrote the manuscript; SW and SSm: coordinated and conducted the studies, gathered data, and participated in data analysis; SSw: analyzed samples and participated in data analysis. DD had primary responsibility for final content. All authors read and approved the final manuscript.

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doi:10.1016/j.metabol.2011.07.015

but to rapid utilization, as the fractional rate of GSH synthesis – measured by the incorporation of labeled cysteine into blood GSH – was increased, rather than decreased, in teenagers with poorly controlled T1D. In contrast, GSH fractional synthesis rate (FSR) was found to be decreased in adults with T2D [8]. Finally, whereas supplementation with cysteine, the limiting precursor amino acid for GSH synthesis, corrects the GSH deficiency associated with undernutrition [9], human immunodeficiency virus infection [10], or T2D [8], GSH pool could not be replenished through dietary supplementation with *N*-acetylcysteine, a donor of cysteine in adolescents with T1D [11].

As hyperglycemia per se is known to enhance the production of reactive oxygen species in mitochondrion [7], we hypothesized that elevation of blood glucose per se could increase the use of GSH to fight oxidative stress, and result in GSH depletion. In earlier studies, we indeed observed that blood GSH was reciprocally related to diabetes control, that is, the higher the hemoglobin A_{1c} (HbA_{1c}), the more profoundly depleted the GSH concentrations [5]. Yet, as the latter studies relied on between-group comparisons, these findings do not directly demonstrate that improving diabetes control would improve GSH status in a given individual.

The question of whether acute improvement in blood glucose control can ameliorate GSH depletion is relevant to the care of these youngsters because GSH depletion may weaken the defense against oxidative stress and thus contribute to the onset of diabetic complications.

We hypothesized that acute normalization of blood glucose might decrease GSH utilization and increase blood GSH concentration. The aim of the current study therefore was to determine whether short-term changes in plasma glucose concentration would alter the rates of GSH utilization and/or affect blood GSH pool in adolescents with poorly controlled T1D.

2. Methods

2.1. Materials

L-[6,6-$^2H_2$]cysteine (98% $^2H_2$) was obtained from Cambridge Isotope Laboratories (Andover, MA) and verified to be sterile and pyrogen free using the limulus lysate assay. The day before each infusion study, tracer solutions were prepared in our pharmacy using aseptic technique by dissolving accurately weighed amounts of labeled cysteine in known volumes of sterile, 0.9% saline; passed through a 0.22-μm Millipore filter (Millipore, Billerica, MA); and stored at 4°C in sterile, sealed vials until infusion.

2.2. Subjects

Ten adolescents who had been followed at the Division of Endocrinology at the Nemours Children's Clinic, Jacksonville, FL, for T1D were recruited after obtaining informed consent from their parents or guardians and assent from the adolescents themselves, according to protocols approved by Wolfson Children's Hospital Institutional Review Board.

2.3. Protocol design

Each patient was admitted to the Clinical Investigational Unit at Wolfson Children's Hospital (Jacksonville, FL) on 2 separate nights, a few days apart, and underwent 2 separate isotope infusions under conditions of either near normoglycemia or moderate hyperglycemia in randomized order.

The evening before each infusion day, the subjects' subcutaneous insulin doses were adjusted so as to bring blood glucose into a target range of either approximately 220 to 280 mg/dL (hyperglycemic day) or approximately 80 to 160 mg/dL ("normoglycemic" day); and blood glucose was maintained overnight in the desired range using subcutaneous boluses of Novolog insulin (Novo Nordisk, Princeton, NJ). An intravenous catheter was inserted after dinner and kept patent using a slow drip of isotonic sodium chloride solution. The intravenous line was used to monitor blood glucose at 60-minute intervals throughout the night and at 30-minute intervals throughout the following morning until the end of the isotope infusion. Patients remained fasting after 10:00 PM until completion of the isotope infusion at 1:00 PM on the following day.

At around 7:00 AM, after numbing the skin with an anesthetic cream, 2 short intravenous catheters were placed: one in a forearm vein for isotope and insulin infusions, and the other one in a superficial vein of the contralateral hand for blood sampling. During the sampling period, the hand was placed in a heating pad at 60°C to obtain arterialized venous blood samples [12]. During the isotopic infusions, blood glucose was maintained in the desired range (either hyper- or normoglycemic) using an intravenous infusion of regular insulin, as in earlier studies [5,11,13]. At 8:00 AM, a primed, continuous infusion of L-[3,3-$^2H_2$]-cysteine (5 μmol kg^{-1} h^{-1}) was started and continued for 5 hours.

Ten milliliters of arterialized venous blood was obtained at –15 and 0 minute, and at 30-minute intervals between 120 and 300 minutes of the isotope infusion for measurement of blood hematocrit and plasma glucose concentration and for the concentration and $^2H_2$-enrichment of erythrocyte free cysteine and GSH. Additional blood samples were obtained for HbA_{1c} and fructosamine measurement. At 1:00 PM, the isotope infusion was discontinued; and subjects received appropriate doses of subcutaneous insulin, ate lunch, and were sent home on their routine home insulin programs. Within 2 weeks, subjects returned to the Clinical Research Center (CRC) for identical studies sustaining the blood glucose either in the hyper- or euglycemic range.

2.4. Analytical procedures

The concentration and $^2H_2$-enrichment of erythrocyte cysteine and GSH were determined by gas chromatography–mass spectrometry as described earlier [14,15]. The typical coefficients of variation for the determination of $^2H_2$ enrichments were 2.9% and 0.4% for GSH and cysteine, respectively [16]. Blood glucose was measured using a Beckman (Fullerton, CA) glucose analyzer via a glucose oxidase method [16]. Hemoglobin A_{1c} was measured using a DCA 2000 (Siemens Heathcare, Deerfield, IL) [17]; and fructosamine concentrations, by high-performance liquid chromatography [18].

2.5. Calculations

The GSH FSR (% d^{-1}) was determined from the incorporation of $^2H_2$-cysteine into red blood cell GSH: FSR = 100 × 24 × ($\Delta E_{GSH}/\Delta t$)/$Erbc_{cys}$, where $Erbc_{cys}$ is $^2H_2$-cysteine in red blood cell at steady state (mol% excess), $\Delta E_{GSH}/\Delta t$ is the slope (MPE h^{-1}) of the regression line describing the rise in red blood cell $^2H_2$-GSH enrichment (MPE) as a function of time (h), and 24 and 100 convert FSR to percentage per day [5,10]. The GSH absolute synthesis rate (ASR, μmol L^{-1} d^{-1}) was calculated as ASR = FSR × [GSH], where [GSH] is GSH concentration, expressed in micromoles per liter of whole blood.

Cysteine appearance rate (Ra, μmol kg^{-1} h^{-1}) was calculated as Ra = i × [(Ei/Erbc) – 1], where i is the rate of L-[3,3-$^2H_2$]-cysteine infusion (μmol kg^{-1} h^{-1}) and Ei and Erbc are the $^2H_2$-enrichments (mol% excess) in infusate and erythrocyte cysteine at steady state, respectively [5,11].

2.6. Statistical analysis

Results are expressed as means ± SE. Parameters were compared between treatments using repeated-measures analysis of variance and paired t tests. Significance was established at $P < .05$. The study was powered to detect a 20% change in GSH concentration of FSR between study days with a power ($1 - \beta$) of 80% and an α risk of 5%.

3. Results

Patients with T1D were included if they met the following criteria: (1) age between 14 and 18 years; (2) duration of diabetes of 6 months or more; (3) absence of any detectable diabetic complication; and (4) poor glycemic control over the previous 3- to 6-month period, as defined by an HbA_{1c} greater than 8.0%. Selected clinical characteristics of the population enrolled are listed in Table 1.

As per study design, plasma glucose was maintained at 244 ± 9 and 118±7 mg/dL ($P < .0001$) on the hyper- and euglycemic day, respectively (Table 2), and was near steady state over the last 3 hours of isotope infusion, with average coefficients of variation of 7.3% and 14.3% on the hyper- and normoglycemic day, respectively. None of the patients experienced hypoglycemia or ketosis during the infusion period. Their average blood glucose control had been similar before each infusion day, as attested by the unchanged fructosamine concentrations (420 ± 29 and 409 ± 28 μmol/L on the hyper- and euglycemic day, respectively; $P = .501$).

Table 1 – Selected clinical characteristics of patients enrolled

Age, y	16.9 ± 1.5
Sex, male/female	7/3
Tanner stage	IV-V
Height, cm	175 ± 2
(Z-score)	(0.4 ± 0.4)
Weight, kg	65 ± 3
(Z-score)	(0.4 ± 0.3)
Body mass index, kg/m^2	21.7 ± 0.6
(Z-score)	(0.2 ± 0.2)
Duration of diabetes, y	8.5 ± 1.9
Insulin regimen: pump/multiple daily injections	7/3
HbA_{1c}, %	9.2 ± 0.5

Values are means ± SE.

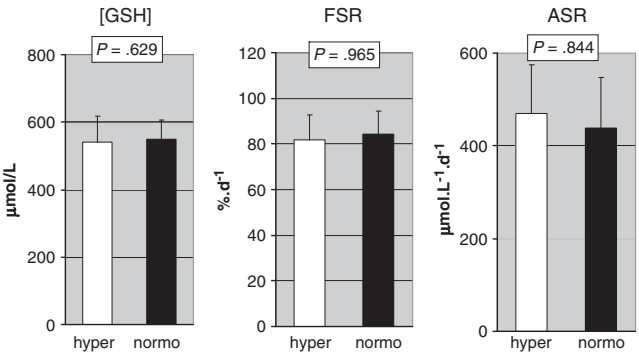

Fig. 1 – Blood GSH concentrations, FSR, and ASR in adolescents with T1D under conditions of short-term hyperglycemia and near normoglycemia; values are means ± SE.

Erythrocyte GSH concentration did not differ between the hyperglycemic day (540.6 ± 77.2 μmol/L) and the euglycemic day (550.8 ± 56.3 μmol/L, $P = .921$, Fig. 1). Glutathione FSR was 81.7% ± 11.2% d^{-1} on the hyperglycemic day and 84.5% ± 10.0% d^{-1} on the euglycemic day ($P = .965$, Fig. 1). Similarly, GSH ASR remained unaltered (469 ± 105 vs 438 ± 110 μmol L^{-1} d^{-1} on the hyper-and euglycemic day, respectively; $P = .844$). The change in blood GSH concentrations between the hyper- and euglycemic study day did not correlate with the change in plasma glucose ($r^2 = 0.037$; not significant). Similarly, cysteine Ra did not differ between the hyper- and the euglycemic day (58.6 ± 5.3 and 55.5 ± 2.7 μmol kg^{-1} h^{-1}, respectively; $P = .37$).

4. Discussion

To the best of our knowledge, the current study is the first to assess the effect of short-term changes in blood glucose control on rates of blood GSH synthesis in adolescents with T1D. Although blood GSH reflects the synthesis of GSH inside erythrocytes, the maintenance of erythrocyte GSH may be relevant to overall GSH homeostasis – and, consequently, tissue oxidative stress – because blood GSH synthesis accounts for more than 10% of whole-body GSH synthesis [19] and changes in erythrocyte GSH parallel changes in other tissues such as gut mucosa [20] or retina [21] in animal models. Therefore, blood GSH depletion may reflect GSH depletion in tissues such as vascular endothelium, where impaired antioxidant plays a role in diabetes complications [22].

From a theoretical standpoint, glucose could have 2 opposite effects on blood GSH. On one hand, a small fraction of cell glucose enters the pentose phosphate pathway and thus produces nicotinamide adenine dinucleotide phosphate (NADPH), a key coenzyme required for the regeneration of reduced GSH from oxidized glutathione (GSSG): elevation of glucose concentration may thus preserve GSH concentrations.

Table 2 – Selected biological parameters in study population on the hyperglycemic and euglycemic study day

	Hyperglycemia	Normoglycemia	P [a]
Fructosamine, μmol/L	431 ± 32	409 ± 28	.702
Plasma glucose, mg/dL	246 ± 8	118 ± 7	.000001
Intravenous insulin delivered, U/5 h	3.4 ± 0.8	5.5 ± 1.5	.117
Hematocrit, %	41.3 ± 1.0	40.9 ± 1.0	.877
Hemoglobin, g/dL	13.8 ± 0.4	13.5 ± 0.4	.762
Serum creatinine, mg/dL	0.9 ± 0.04	0.8 ± 0.04	.025
Serum albumin, g/dL	3.6 ± 0.1	3.7 ± 0.1	.530
Serum osmolality, mOsm/L	280 ± 1	278 ± 1	.910
Blood CO_2	25.3 ± 0.4	25.3 ± 0.5	.488

Values are means ± SE of 10 subjects.
[a] Student *t* test: hyper- vs normoglycemia.

On the other hand, several other pathways of glucose utilization can lead to GSH depletion. First, glycolysis, the main pathway of glucose utilization, indeed produces nicotinamide adenine dinucleotide (NADH); and oxidation of NADH in mitochondrion mainly produces adenosine triphosphate, as well as small amounts of superoxide, a free radical generated as a byproduct of glycolysis. Therefore, elevation of plasma glucose can have a prooxidant effect [23]. As erythrocytes lack mitochondria, the latter source of free radicals is unlikely to play a role in the regulation of red blood cell GSH. However, elevation of glucose concentration increases the activity of the sorbitol pathway in red blood cells; this minor pathway converts glucose to sorbitol, a polyol considered a metabolic "dead end." Aldose reductase, the key enzyme of this pathway, uses NADPH as its coenzyme. As aldose reductase is insulin independent, its activity is entirely dependent on ambient glucose concentrations; so when blood glucose is in the reference range, a minimal fraction of glucose enters that pathway. In contrast, under conditions of chronic hyperglycemia, activity of the polyol pathway increases dramatically and uses large amounts of NADPH, thus competing with the pentose pathway for scarce NADPH stores [24]. Accordingly, inhibition of aldose reductase was found to enhance GSH concentration in erythrocytes from patients with T2D [25].

The main finding of the current study was the inability of an acute improvement in blood glucose control to restore GSH. Few studies specifically addressed the role of elevation of glucose per se on GSH. In vitro, exposing K562 cells in culture to high glucose concentrations (27 mmol/L) in the incubation medium depleted intracellular GSH concentration [26]. In the nondiabetic population, Menon et al [27] reported an inverse correlation between fasting blood glucose and blood GSH concentration. In healthy adult volunteers, van der Crabben et al [28] showed that acute elevation of blood glucose decreased GSH FSR, but failed to alter GSH concentration. In adults with T1D, continuous monitoring of subcutaneous glucose concentration revealed that blood GSH increased in the postprandial period, at a time when blood glucose increased, and decreased in the postabsorptive period [29]. Although the rise in GSH occurred at the time of hyperglycemia in the latter study, it is difficult to attribute the change to glucose per se because the postprandial state is associated with increased availability of amino acid precursors such as cysteine, glycine, and glutamate used for GSH synthesis. Similarly, even though blood GSH concentration decreases during fasting [30], the slight decline in blood glucose associated with fasting may not be the main determining factor.

Treatment of patients with an antidiabetic agent for 6 months resulted in the restoration of γ-glutamylcysteine synthetase activity, the concentration of GSH, and the thiol transport in isolated erythrocytes from adult patients with T2D [31]. In T1D, blood GSH was higher after a year of insulin treatment compared with the low values observed at onset [32]. We are not aware of studies addressing the effect of short-term alterations in blood glucose on GSH in T1D or the effect of insulin per se on GSH homeostasis. Recently, El-Osta et al [33] reported that changes induced by hyperglycemia in the expression of nuclear factor–κB and markers of inflammation in endothelial cells persisted several days after establishment of normal glycemia; and inflammation and oxidative stress are clearly related [34]. We speculate that the reason why we failed to observe any effect of acute normalization of blood glucose was the short duration of the experiment, as normoglycemia was maintained for less than 12 hours in adolescents that had been in a state of chronic hyperglycemia for several months, as attested by their elevated glycohemoglobin. The effect of acute changes in blood glucose might have been different in a group of patients with a better long-term glucose control.

In addition, fluctuations in blood glucose may be more powerful determinants of oxidative stress than the average quality of blood glucose control. In adult patients with T2D [34], urinary excretion of 8-iso-$PGF_{2\alpha}$, a marker of oxidative stress, was strongly correlated with acute glucose fluctuations, expressed as the mean amplitude of glycemic excursions, which has been described by Service et al [35]. In contrast, no relationship was observed when urinary 8-iso-$PGF_{2\alpha}$ excretion rates were plotted against main markers of sustained hyperglycemia (HbA_{1c} and mean daily glucose concentrations).

In summary, in the current report, acute adjustments of blood glucose over a 12-hour period in the postabsorptive state failed to alter blood GSH stores and kinetics in a group of adolescents with poorly controlled T1D. The strength of the current report stems from its paired study design and from the use of stable isotope methods to assess not only GSH concentration but GSH synthesis rates as well. The study obviously suffers from limitations. For instance, (1) only patients who were in poor control were enrolled, so the

findings cannot be extrapolated to well-controlled T1D; (2) as blood glucose was altered over a few hours, the effect of sustained changes in glucose control was not addressed; and (3) whether elevated blood glucose and insulin deficiency per se have separate effects on GSH remains to be explored.

Despite such limitations, the current findings nevertheless are of potential relevance to the care of young patients with diabetes because oxidative stress and, therefore, GSH depletion contribute to the onset of diabetic complications. The current findings indeed suggest that short-term manipulation of blood glucose may be ineffective, and alternate strategies remain to be developed to prevent oxidative stress and potentially delay the onset of complications in T1D.

Funding

This work was supported, in part, by grants from the Juvenile Diabetes Research Foundation (1-2006-259 627) and the Nemours Research Programs (Jacksonville, FL).

Acknowledgment

We thank the patients and families for their participation in these demanding studies. We acknowledge the support from our colleagues Drs Larry Fox, Priscilla Gagliardi, and Robert Olney for their help in recruiting the patients. We are grateful for Bernice Rutledge, RN, and the expert nursing team at Wolfson Children's Hospital for their outstanding care of the patients.

Conflict of Interest

None of the authors had any conflict of interest.

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