Available online at www.sciencedirect.com
ScienceDirect
Metabolism Clinical and Experimental 60 (2011) 99 – 106
Metabolism
Clinical and Experimental
www.metabolismjournal.com

Erythrocyte glutathione concentration and production during hyperinsulinemia, hyperglycemia, and endotoxemia in healthy humans

Saskia N. van der Crabben[a,*,1], Michiel E. Stegenga[b,1], Regje M.E. Blümer[a], Mariëtte T. Ackermans[c], Erik Endert[c], Michael W.T. Tanck[d], Mireille J. Serlie[a], Tom van der Poll[b], Hans P. Sauerwein[a]

[a]*Department of Endocrinology and Metabolism, Academic Medical Center, 1100 DD Amsterdam, The Netherlands*
[b]*Center for Experimental and Molecular Medicine, Academic Medical Center, 1100 DD Amsterdam, The Netherlands*
[c]*Laboratory of Endocrinology and Radiochemistry, Department of Clinical Chemistry, Academic Medical Center, 1100 DD Amsterdam, The Netherlands*
[d]*Department of Clinical Epidemiology, Biostatistics and Bioinformatics, Academic Medical Center, 1100 DD Amsterdam, The Netherlands*

Received 22 February 2010; accepted 3 August 2010

Abstract

In diabetes mellitus and sepsis, low erythrocyte glutathione (GSH) concentrations are found. Whether this is caused by lowered GSH production has not been clarified. To obtain insight in the relationship between erythrocyte GSH concentrations and GSH production, GSH kinetics were measured in healthy male volunteers during 4 different clamps (low-dose or medium-dose insulin [100 or 400 pmol/L] and euglycemia or hyperglycemia [5 or 12 mmol/L]) in a control setting (n = 6; all 4 clamps in the same subject) or after systemic administration of lipopolysaccharide (to mimic sepsis) (4 groups of n = 6; each clamp in a different subject). Hyperinsulinemia decreased erythrocyte GSH concentration (P = .042), but did not affect fractional synthetic rate (FSR) of GSH. Hyperglycemia did not affect erythrocyte GSH concentration, but decreased FSR of GSH (P = .025). Lipopolysaccharide decreased erythrocyte GSH concentration (P < .001), but increased FSR of erythrocyte GSH (P = .035). Depending on the metabolic circumstances, we found either stable GSH concentrations with lower production rates or decreased levels with either no change or an increase in production rate. Based upon these data, it seems inappropriate to infer conclusions about changes in synthesis rate of GSH from changes in its concentration.

1. Introduction

The tripeptide glutathione (γ-glutamyl-cysteinyl-glycine; GSH) is the main antioxidant defense system in humans. Glutathione neutralizes reactive oxygen species (ROS) and thereby plays a pivotal role in the defense against oxidative stress. Oxidative stress indicates an excess of ROS due either to overproduction of ROS or to a depletion of GSH, leading to damage of protein, lipids, and DNA [1]. Glutathione erythrocyte concentrations are depleted in several disease states, such as type 1 diabetes mellitus (DM1), type 2 diabetes mellitus, and sepsis [2-6]. Deregulation of GSH metabolism is seen as an important contributor to the development of complications in these diseases [7].

A decreased GSH erythrocyte concentration is the consequence of a mismatch between GSH production and GSH use or excessive oxidized GSH (GSSG) production. Excessive GSSG in the erythrocyte is actively transported outside the cell, thereby decreasing the total GSH concentration. Decreased GSH production can be due to shortage of its precursors: glutamate, glycine, and cysteine. Cysteine is thought to be the most critical amino acid, as it is rate limiting in GSH synthesis [7].

It has been reported that, in several other diseases, supplements of cysteine can stimulate GSH synthesis [8-10]. Studies on the relationship between erythrocyte GSH concentrations and its production are therefore of importance because a lower synthesis rate may be susceptible to

* Corresponding author. University Medical Center Utrecht (Wilhelmina Children's Hospital), DBG Department of Clinical Genetics, PO Box 85090, 3508 AB Utrecht, The Netherlands. Tel.: +31 88 7553800; fax: +31 88 7553801.
E-mail address: s.n.vandercrabben@umcutrecht.nl (S.N. van der Crabben).
[1] Contributed equally to this work.

0026-0495/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2010.08.003

treatment with cysteine [11]. The scarce data on GSH kinetics suggest however that a lower erythrocyte GSH concentration is not always due to a lower rate of GSH production. In poorly controlled patients with DM1, erythrocyte GSH concentrations are low despite an elevated fractional synthetic rate (FSR) [5]. In septic children, FSR of erythrocyte GSH, as well as its concentration, is decreased [6]. Under these circumstances, the disturbances in glucose metabolism, which are almost always present during sepsis [12]; a direct effect of the infection/sepsis; or a combination of both factors might influence the relationship between erythrocyte GSH production and its concentration.

To explore the relationship between erythrocyte GSH concentration and GSH production in adults under different metabolic circumstances, we studied FSR of erythrocyte GSH and GSH concentration using stable isotopes during a hyperinsulinemic (100 and 400 pmol/L), euglycemic (5 mmol/L), or hyperglycemic (12 mmol/L) clamp in healthy male volunteers either in a control setting or after administration of lipopolysaccharide (LPS; the toxic component of the outer membrane of gram-negative bacteria) to mimic a state of sepsis.

2. Patients and methods

The study was approved by the Medical Ethical Committee of the Academic Medical Center in Amsterdam, and all subjects gave written informed consent.

2.1. Volunteers and study groups

Thirty healthy, nonsmoking, male volunteers were included. None of them used medication, had an infection in the preceding 3 months, was overweight or obese (defined as a body mass index >25 kg/m^2), or had a positive family history of diabetes. All volunteers had normal plasma values of fasting glucose, erythrocyte sedimentation rate, complete blood count, lipid profile, creatinine, and liver enzymes; and all had a normal oral glucose tolerance test according to the American Diabetes Association criteria [13]. Volunteers were studied on 4 occasions: during euglycemia (5 mmol/L) or hyperglycemia (12 mmol/L) in combination with either a low-dose (100 pmol/L) or a medium-dose (400 pmol/L) plasma insulin level.

In the control group (not receiving LPS), volunteers (n = 6) were studied during all 4 clamps in a crossover design in random order. Because we considered it not feasible to administer LPS twice to the same subjects, the clamps in LPS-injected humans were performed in 4 separate groups, each consisting of 6 volunteers (total, n = 24). Volunteers were randomly assigned to each clamp group. For 3 days before the study, all volunteers were asked to refrain from vigorous exercise. The present study was part of a study on the differential effects of plasma glucose and insulin concentrations on several parameters relevant for critical ill patients [14-18]. The data on GSH metabolism have not been published earlier.

2.2. Protocol in the control and the LPS group

At 7:45 AM in the LPS and 8:45 AM in the control group, a catheter was inserted into an antecubital vein for infusion of [3,3-$^2H_2$]cysteine, insulin, somatostatin, glucagon, glucose (10% or 20%), and LPS (the difference in time was due to a slight difference in study protocol). Another catheter was inserted retrogradely into a contralateral hand vein kept in a thermoregulated (60°C) Plexiglas box for sampling of arterialized venous blood (Fig. 1).

At T = 0:00 hour (15 minutes after insertion of the catheters), a blood sample was drawn for determination of background enrichment and GSH concentration. Thereafter, a primed-continuous infusion of [3,3-$^2H_2$]cysteine (prime: 5.0 μmol/kg; continuous: 0.08 μmol kg^{-1} min^{-1}; >98% pure and >99% enriched; Cambridge Isotope Laboratories, Cambridge, MA), together with somatostatin (250 μg·h; Somatostatine-ucb, UCB Pharma BV, Breda, the Netherlands), glucagon (1 ng·kg^{-1}·min^{-1}; GlucaGen, Novo Nordisk, Alphen a/d Rijn, the Netherlands), insulin (Actrapid, Novo Nordisk, Alphen a/d Rijn, the Netherlands) at a rate of 10 or 40 mU/m^2 body surface area per minute (aimed for plasma insulin concentrations of 100 or 400 pmol/L, respectively), and glucose (10% or 20%) at a variable rate to obtain euglycemia (5 mmol/L) or hyperglycemia (12 mmol/L), were started and continued for 6 hours in the control group and for 8 hours in the LPS group. Somatostatin was infused to prevent differences in insulin and glucagon concentration between the LPS and the control group during the clamps. Insulin was infused to obtain the esteemed concentration of 100 or 400 pmol/L. As glucagon is important for hepatic glucose production, this was infused in a basal concentration.

In the LPS group, the euglycemic medium-dose insulin clamp had a slightly different insulin infusion regimen after an interim analysis of glucose and insulin in 3 subjects had indicated that during the low-dose and the medium-dose hyperglycemic clamps, the rate of infusion of somatostatin appeared insufficient to achieve complete suppression of endogenous insulin production between T = 5:00 and T = 7:00 (2 and 4 hours after LPS administration) (data not shown and not included in further analyses). To keep distinct lower and high insulin concentrations from T = 5:00 onward, we included 6 new subjects in whom insulin infusion in the medium-dose euglycemic clamp was increased at this time point to 100 mU/m^2 body surface area per minute.

At T = 3:00 hours (after 3 hours of clamping), purified, lyophilized LPS (from *Escherichia coli*, lot G; United States Pharmacopeial Convention, Rockville, MD) was administered to the LPS group by intravenous bolus (4 ng/kg) after reconstitution in sterile water for injection.

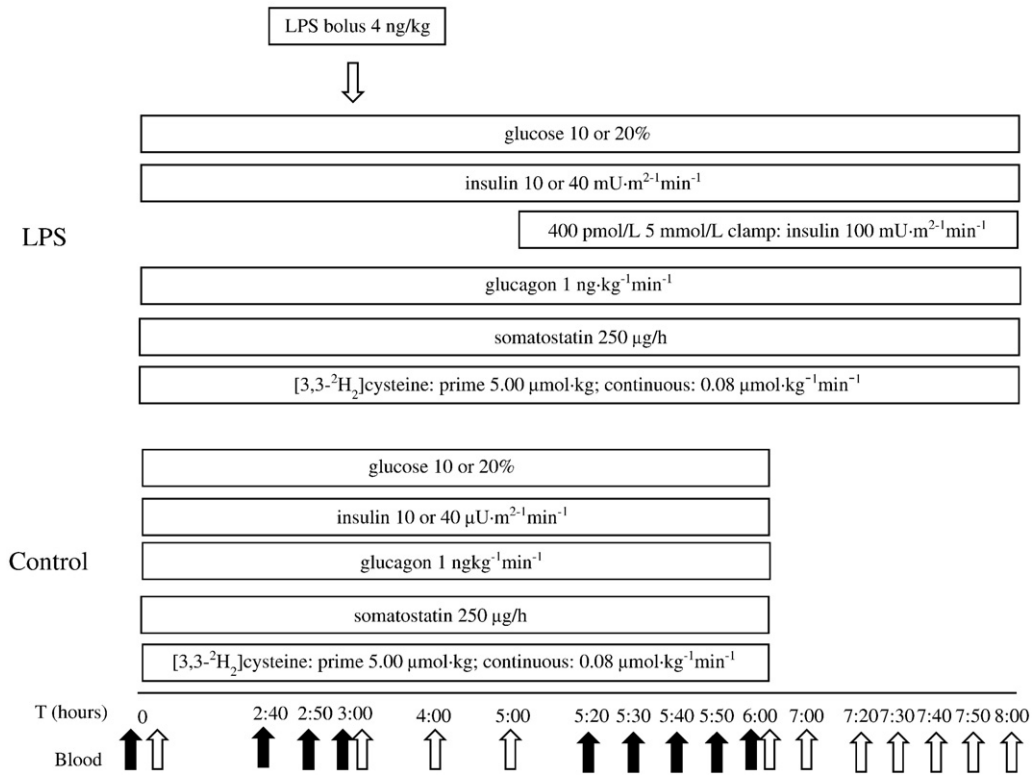

Fig. 1. Experimental design for the clamp studies in the control and LPS groups. $T = 0$ hour is 8:00 AM in the LPS group and 9:00 AM in the control group. Black arrow = control group; white arrow = LPS group.

2.3. Glucose and insulin concentration

Plasma glucose was measured every 5 minutes at bedside from $T = 0{:}00$ until $T = 6{:}00$ in the control group and until $T = 8{:}00$ in the LPS group. In the control group, from $T = 2{:}40$ until $T = 3{:}00$ and from $T = 5{:}40$ until $T = 6{:}00$, blood samples were drawn every 10 minutes for determination of the concentration of plasma insulin. In the "Results" section, the mean values of these 3 measurements are used.

For determination of plasma insulin concentration in the LPS group, blood samples were drawn at $T = 0{:}00$ and hourly from $T = 3{:}00$ until $T = 8{:}00$. Measurements of these parameters were done as described before [19].

2.4. GSH enrichment

At $T = 0{:}00$, one blood sample was drawn for determination of background enrichment in both groups. From $T = 5{:}20$ until $T = 6{:}00$ in the control group and from $T = 7{:}20$ until $T = 8{:}00$ in the LPS group, 5 blood samples were drawn for measurement of [3,3-$^2H_2$]cysteine and GSH enrichment. The mean values of these 5 samples will be presented as GSH concentration at $T = 6{:}00$ and $T = 8{:}00$. For calculation of the FSR of GSH, all 5 samples were used.

For measurement of [3,3-$^2H_2$]cysteine and GSH enrichment, 3 mL of venous blood was collected in K-EDTA tubes, mixed, and divided into 2 portions. One portion was frozen at −80°C and used for the analysis of whole blood GSH concentration and isotopic enrichment. Glutathione is produced in the erythrocyte, and only about 0.5% of the whole blood GSH concentration is derived from plasma. Therefore, measuring GSH concentration as well as its isotopic enrichment in whole blood is a direct measure for the erythrocyte concentration and enrichment.

The other tube was used for measurement of 3,3-$^2H_2$-cysteine enrichment in the erythrocytes. For a correct estimate of the FSR, it is necessary that the precursor pool enrichment is measured in the pool where synthesis takes place. Glutathione is synthesized in erythrocytes; and therefore, precursor pool enrichment in pure erythrocytes should be used. Furthermore, 3,3-$^2H_2$-L-cysteine enrichment of cysteine in plasma is much higher than enrichment in erythrocytes. For these 2 reasons, the enrichment of d_2-cysteine should be measured in washed erythrocytes. Therefore, after immediate centrifuging for 10 minutes at 1500*g* at 4°C, the upper liquid level was marked with a line on the outside of the tube. After removal of the plasma, cold sodium chloride (0.9% wt/vol) was added until the line on the tube and mixed well to resuspend the cells. After centrifuging for 10 minutes at 1500*g*, the supernatant was removed again. This washing step was repeated twice, and then the tube was filled up to the marking line with distilled water and stored at −80°C.

Isotopic enrichments of cysteine and GSH were analyzed as described by Capitan et al [20] with minor modifications.

For the determination of the 3,3-$^2H_2$-cysteine enrichments, a calibration curve was used; for the determination of the d_2-GSH enrichment, a slope of unity was assumed. The concentration of GSH was determined by an internal standard method, using homoGSH as an internal standard, as described before [21].

Gass chromatography (GC)–mass spectrometry analysis was carried out on a Hewlett Packard GC/mass selective detector system (Model 6890/5973; HP, Palo Alto, CA). The temperature of the GC injector was 250°C for cysteine and 270°C for GSH. The column used was an Agilent HP5ms column (internal diameter, 0.25 mm; *df*, 0.25 μm) of 30 m for the cysteine and 10 m for the GSH (Agilent, Palo Alto, CA). Cysteine injections (1 μL) were made in the splitless mode; GSH injections (0.5 μL) were made in the split mode (ratio 10:1). Helium was used as carrier gas. The mass spectrometer was used in the electron impact mode (electron energy, 70 eV; source temperature, 230 °C; quad temperature, 150°C). Data acquisition and integration were done using the HP Chemstation software (Version D.00.01, HP). The ion fragments of the *N*,*S*-ethoxycarbonyl methyl ester derivative of cysteine and GSH were recorded in the selective ion monitoring mode at *m*/*z* 220 and *m*/*z* 222 for cysteine and 3,3-$^2H_2$-cysteine, *m*/*z* 363 for GSH and hGSH, and *m*/*z* 365 for d_2-GSH.

2.5. Calculations

To correct for potential differences in baseline values, the change in GSH concentration during all 4 clamps (T = 6:00 compared with T = 0:00 in the control group and T = 8:00 compared with T = 0:00 in the LPS group) was calculated and expressed as the relative (percentage) difference.

The tracer-tracee ratio (TTR) of 3,3-$^2H_2$-cysteine was calculated from the calibration graph where the measured TTR $\left(\mathrm{TTR}_{^2\mathrm{H2-Cysteine}} = \frac{\text{peak area 222}}{\text{peak area 220}}\right)$ for the standards is plotted against the theoretical TTR.

Tracer-tracee ratio of enrichment of GSH:

$$\mathrm{TTR}_{d_2-\mathrm{GSH}} = \frac{\text{peak area 365}}{\text{peak area 363}}$$

Tracer-tracee ratio values were corrected for natural enrichment by subtracting the TTR before infusion (baseline value) from the ones after infusion.

The FSR of GSH was calculated as:

$$\mathrm{FSR}_{\mathrm{GSH}} = 100 \times 24 \times \frac{\frac{\Delta \mathrm{TTR}_{d_2-\mathrm{GSH}}}{\Delta t}}{\mathrm{TTR}_{3,3-{}^2\mathrm{H2-cyst}}}$$

$\frac{\Delta \mathrm{TTR}_{d_2-\mathrm{GSH}}}{\Delta t}$ is the slope of the linear regression line of $\mathrm{TTR}_{d_2\text{-GSH}}$ vs t (in hours), and $\mathrm{TTR}_{3,3\text{-}^2\mathrm{H2\text{-}cyst}}$ is the plateau enrichment of 3,3-$^2H_2$-cysteine; 24 converts from per hour to per day, and 100 converts a fraction of unity to a percentage.

Table 1
Tracer-tracee ratio of [3,3-$^2H_2$]cysteine in erythrocytes and d_2-GSH in whole blood in the control and the LPS group during the low-dose (100 pmol/L) and the medium-dose (400 pmol/L) euglycemic (5 mmol/L) or hyperglycemic (12 mmol/L) clamps

			Control group						LPS group				
		T	5:20	5:30	5:40	5:50	6:00	7:20	7:30	7:40	7:50	8:00
TTR (%), 3,3-$^2H_2$-cysteine	Low dose	Euglycemic	0.85 ± 0.10	0.85 ± 0.09	0.86 ± 0.09	0.87 ± 0.09	0.86 ± 0.09	0.80 ± 0.13	0.81 ± 0.17	0.81 ± 0.16	0.82 ± 0.18	0.82 ± 0.19
		Hyperglycemic	0.86 ± 0.08	0.85 ± 0.10	0.87 ± 0.09	0.87 ± 0.08	0.88 ± 0.07	0.68 ± 0.13	0.71 ± 0.12	0.71 ± 0.09	0.71 ± 0.12	0.69 ± 0.09
	Medium dose	Euglycemic	0.83 ± 0.10	0.83 ± 0.09	0.84 ± 0.09	0.85 ± 0.09	0.86 ± 0.09	0.70 ± 0.05	0.71 ± 0.05	0.71 ± 0.03	0.72 ± 0.04	0.71 ± 0.05
		Hyperglycemic	0.84 ± 0.10	0.85 ± 0.10	0.86 ± 0.09	0.87 ± 0.10	0.87 ± 0.10	0.65 ± 0.13	0.66 ± 0.13	0.64± 0.12	0.67 ± 0.14	0.66 ± 0.12
TTR (%) d_2-GSH	Low dose	Euglycemic	0.78 ± 0.20	0.78 ± 0.19	0.82 ± 0.21	0.85 ± 0.22	0.84 ± 0.23	2.16 ± 0.27	2.22 ± 0.31	2.26 ± 0.27	2.32 ± 0.51	2.56 ± 0.30
		Hyperglycemic	0.74 ± 0.22	0.75 ± 0.23	0.77 ± 0.24	0.79 ± 0.22	0.77 ± 0.22	0.81 ± 0.21	0.84 ± 0.22	0.85 ± 0.22	0.87 ± 0.24	0.88 ± 0.26
	Medium dose	Euglycemic	0.71 ± 0.16	0.73 ± 0.19	0.73 ± 0.19	0.78 ± 0.18	0.81 ± 0.19	0.93 ± 0.10	0.96 ± 0.12	1.01 ± 0.13	1.04 ± 0.14	1.05 ± 0.11
		Hyperglycemic	0.68 ± 0.18	0.64 ± 0.15	0.73 ± 0.15	0.71 ± 0.17	0.75 ± 0.17	0.74 ± 0.04	0.78 ± 0.03	0.78 ± 0.05	0.81 ± 0.03	0.82 ± 0.03

Data are expressed as mean ± SD; n = 6 in each group.

2.6. Statistical analysis

All data are presented as median (range), except the data in Table 1 (mean ± SD). Volunteer characteristics were compared using a Kruskal-Wallis test. To measure the effect of time on erythrocyte GSH concentration, GSH concentrations at the end of the clamp (T = 6:00 in the control and T = 8:00 in the LPS group) were compared with values at the beginning of the clamp (T = 0:00) using a Wilcoxon signed rank test. All variables were rank transformed for subsequent analysis. Within the control group, relative (percentage) change of GSH concentration and FSR of GSH from the 4 different clamps was compared using a repeated-measures analysis of variance (ANOVA). Compound symmetry for measurements within patients was assumed.

Within the LPS group, relative (percentage) change of GSH concentration and FSR of GSH from the 4 different clamps was compared using an ANOVA. In case of the existence of an overall effect, the clamps within each group (control or LPS) were compared with the low-dose euglycemic clamp using a post hoc test. To analyze the effect of the medium-dose insulin (400 pmol/L), high glucose (12 mmol/L), LPS, and their interactions, results of all the clamps from the control or LPS group were compared using a repeated-measures ANOVA. Probability values < .05 were considered statistically significant. SPSS statistical software version 12.0.1 (SPSS, Chicago, IL) was used to analyze the data.

3. Results

3.1. Subjects characteristics

There was no difference in age (years) or body mass index (kilograms per square meter) between the control group (n = 6; respectively: 22 [22-23]; 21.7 [21.0-23.4]) and the LPS group (low dose: euglycemic [n = 6]: 23 [20-25]; 22.0 [19.9-23.6] and hyperglycemic [n = 6]: 22 [19-25]; 23.3 [20.2-24.6]; medium dose: euglycemic [n = 6]: 25 [21-27]; 22.1 [20.9-23.7] and hyperglycemic [n = 6]: 22 [18-27]; 21.8 [20.6-24.6]) (P = .249 and .837, respectively).

3.2. Control group glucose and insulin levels

The plasma glucose and insulin levels during all clamps have been published before [14]. In short, plasma glucose levels remained at approximately 5 mmol/L throughout the low-dose and medium-dose insulin euglycemic clamp (both P < .05 compared with the low-dose and medium-dose hyperglycemic clamp). In the low-dose insulin hyperglycemic and the medium-dose insulin hyperglycemic clamp, plasma glucose concentrations rapidly increased during the first hour and from 3 hours onward remained stable at approximately 12 mmol/L.

Plasma insulin concentrations in the low-dose hyperglycemic clamp were modestly higher compared with the low-dose euglycemic clamp (127 ± 19 vs 89 ± 6 pmol/L, respectively; P < .05). In the medium-dose euglycemic and the medium-dose hyperglycemic clamp, target plasma insulin concentrations were reached from T = 3:00 onward (408 ± 61 and 443 ± 34 pmol/L at T = 6:00, respectively) (P < .05 for the difference with either of the low-dose clamps).

3.3. Control group erythrocyte GSH concentration and FSR

At the beginning of the clamps, GSH concentrations were similar. At T = 6:00, erythrocyte GSH concentration had declined most during the medium-dose hyperglycemic clamp (compared with the low-dose euglycemic clamp [P = .002]). The FSR of GSH was lowest during the medium-dose hyperglycemic clamp (compared with the low-dose euglycemic clamp [P = .018]).

3.4. LPS group glucose and insulin levels

The plasma glucose and insulin levels during all clamps have been published before [16]. In short, throughout the low-dose and the medium-dose euglycemic clamps, plasma glucose levels remained at approximately 5 mmol/L. In the low-dose and medium-dose hyperglycemic clamps, plasma glucose levels rapidly increased during the first hour; and from T = 2:00 onward, plasma glucose levels remained stable at approximately 12 mmol/L, except for a temporary small drop between T = 5:00 and T = 7:00 (2 and 4 hours, respectively, after LPS injection) (P < .001 for the difference between the euglycemic and either of the 2 hyperglycemic clamps).

Plasma insulin concentrations were statistically different between the low-dose and the medium-dose clamps, except between T = 5:00 and T = 7:00 when insulin concentrations increased in all clamps except the low-dose euglycemic clamp (P < .001 for all time points except from T = 5:00 until T = 7:00). Values reached during these increases were higher than the attempted values of 100 and 400 pmol/L.

3.5. LPS group erythrocyte GSH concentration and FSR

At the beginning and the end of the clamps, GSH concentrations between clamps did not differ. Fractional synthetic rate of GSH was equal in all clamps.

3.6. Control vs LPS group erythrocyte GSH concentration and FSR

At T = 0:00, there was no difference in GSH concentration between the control and the LPS groups. Erythrocyte GSH concentration in both the control and the LPS groups was lowered by high insulin concentration (P = .042), whereas FSR of GSH was unaffected. Lipopolysaccharide decreased erythrocyte GSH concentration (P < .001), but increased FSR of erythrocyte GSH (P = .035). Hyperglycemia did not affect erythrocyte GSH concentration but decreased FSR of GSH (P = .025).

4. Discussion

It is generally assumed that a decreased erythrocyte GSH concentration is due to a decreased erythrocyte GSH production. The results of the present study show for the first time in adults that erythrocyte GSH concentration can be decreased (by high insulin concentration and LPS), whereas FSR of GSH can be, respectively, unaffected or increased. Moreover, stable erythrocyte GSH concentration (during hyperglycemia) can be accompanied by a decreased FSR of GSH. These differential effects on erythrocyte GSH concentration and production show that a direct relationship between these 2 parameters does not necessarily exist.

The decreasing effect of hyperinsulinemia on erythrocyte GSH concentration, despite the absence of an effect on FSR, indicates increased use of GSH due to excessive ROS or an increased GSSG loss [7]. Considering the lack of mitochondria in erythrocytes, the last option is the most likely. A possible explanation for this decreased GSH concentration might be the (non–insulin-mediated) glucose influx into the cell, leading to an increased flux via the polyol pathway [22]. In the polyol pathway, NADPH is a mandatory cofactor. Glutathione reductase also needs NADPH to reduce GSSG.

If less NADPH is available for glutathione reductase, GSSG concentration rises. Excess GSSG either is actively exported from the cells or reacts with protein sulfhydryls, via a mixed disulfide reaction. This leads to a net loss of total GSH [7]. In general, a decrease of erythrocyte GSH concentration has been seen in all clamps and could be considered an effect of time or an effect of diurnal variation [23].

The lowering effect of LPS on erythrocyte GSH concentrations and the stimulating effect on erythrocyte FSR of GSH in our study in adults are similar to prior findings on GSH concentrations in septic pediatric patients, but contrast with their finding of decreased erythrocyte GSH production [6]. Noteworthy is that, in the latter study, the control group was composed of surgical patients and therefore not healthy controls. Literature is not conclusive on this point, as other (scarce) data indicate that, in animals, endotoxemia has both an increasing as well as a neutral effect on erythrocyte GSH concentration and FSR of erythrocyte GSH [24,25].

An important difference between these studies and our results is the time course of the systemic inflammatory response. Inflammatory reactions in the endotoxemia model are self-limiting within several hours to 1 day, in contrast to responses in septic patients [26]. Another difference between patients and our model used is that patients are often fed enterally and we used fasted subjects. Measurements of the mentioned studies were performed at (maximal) 48 hours after LPS injection, whereas our measurements took place 5 hours after endotoxemia.

The inhibiting effect of hyperglycemia on FSR of erythrocyte GSH in the presence of an unaffected erythrocyte GSH concentration seems to be in contrast with the elevated FSR of GSH and the depleted erythrocyte GSH found in poorly controlled DM1 subjects [5]. However, the reasonably well-controlled DM1 subjects described by Darmaun et al [5] have significantly lower erythrocyte GSH concentrations compared with the healthy volunteers, but an unchanged FSR of GSH.

The unchanged FSR was present despite an increased erythrocyte free cysteine concentration compared with the healthy controls. As a close relationship between GSH production and cysteine concentration is supposed, this is a remarkable finding. Unfortunately, in our study, no data on erythrocyte cysteine concentrations were available. Despite this, we consider the data presented by Darmaun et al [5], although apparently different, to support our conclusion that no direct relationship between erythrocyte GSH concentration and its production can be assumed.

A possible explanation for our different findings could be the short-term glucose deregulation in our subjects vs the chronic glucose deregulation in the diabetic subjects. In addition, a direct effect of the somatostatin or glucagon infusions cannot be ruled out. It has been suggested that a decreased growth hormone production decreases GSH production [11]. Although no study has been published so far to confirm this supposition in human erythrocytes, we cannot exclude an inhibiting effect on the FSR of GSH by somatostatin in our studies. As somatostatin was used in all clamps during the control as well as the LPS setting, this could not have had a significant impact on our conclusions. The exact mechanism leading to this decreased erythrocyte GSH production remains to be established.

The published data on FSR of erythrocyte GSH are equal to previous studies from our group [27,28], but are higher compared with the results of other studies [5,6,10,20]. This difference can be explained by the precursor pool enrichment for FSR determination for which washing of the erythrocytes is essential. Because the FSR is calculated by dividing the increase in GSH enrichment by the precursor pool enrichment, values calculated with TTR in washed erythrocytes will be much higher than reported until now because the denominator is much lower, as enrichment in those studies is higher because of the high isotope concentration in adhering plasma.

A possible confounding factor is the difference in study period between the control and the LPS groups, with a concomitant increase in enrichment in cysteine in the LPS group. However, this possibility is less likely, as when during these 2 hours enrichment of cysteine would have increased because of, for example, recycling of the label, a lower production would have been found instead of the observed increase in production.

As described earlier [16], although we used somatostatin to suppress endogenous insulin and glucagon production during all clamps, we did not achieve complete suppression: from 2 until 4 hours after LPS administration, somatostatin did not completely block hyperglycemia-driven insulin

production in the hyperglycemic clamps. Importantly, before and after this time window, suppression by somatostatin seemed adequate, as indicated by a clear separation between high and low glucose and insulin levels. Apparently, factors in the early inflammation phase interfered with the suppressive effect of somatostatin. Using a higher dose of somatostatin would have been problematic because this can have nausea as an adverse effect. Using the current dose, the combination with endotoxin already induced nausea in 19 of 24 volunteers. Therefore, we chose to adjust the protocol for the 400-pmol/L:5-mmol/L clamp to keep sufficient distinction between glucose and insulin levels in the 4 different clamps.

In conclusion, the results of our study indicate that no direct relationship between GSH concentration and GSH production can be assumed. Distinct metabolic circumstances have a differential effect on GSH concentration and production rate. This is especially important when considering use of supplements in diseases with low GSH concentrations with the aim to increase GSH production rate. Further research to explore the exact mechanism of discrepancies between GSH production and GSH concentrations is necessary for assessing the best therapeutic intervention to prevent oxidative damage that may lead to a decrease in morbidity and mortality from diseases characterized by high oxidative stress.

Acknowledgment

We would like to thank AFC Ruiter, BCE Voermans, and the staff of the Clinical Research Unit of the Academic Medical Center in Amsterdam for their indispensable help during the experiments. This study was supported by a grant from the Dutch Diabetes Research Foundation (2002.00.008).

References

[1] Newsholme P, Haber EP, Hirabara SM, Rebelato EL, Procopio J, Morgan D, Oliveira-Emilio HC, Carpinelli AR, Curi R. Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity. J Physiol 2007; 583:9-24.

[2] Dincer Y, Akcay T, Alademir Z, Ilkova H. Effect of oxidative stress on glutathione pathway in red blood cells from patients with insulin-dependent diabetes mellitus. Metabolism 2002;51:1360-2.

[3] Seghrouchni I, Drai J, Bannier E, Riviere J, Calmard P, Garcia I, Orgiazzi J, Revol A. Oxidative stress parameters in type I, type II and insulin-treated type 2 diabetes mellitus; insulin treatment efficiency. Clin Chim Acta 2002;321:89-96.

[4] Murakami K, Kondo T, Ohtsuka Y, Fujiwara Y, Shimada M, Kawakami Y. Impairment of glutathione metabolism in erythrocytes from patients with diabetes mellitus. Metabolism 1989;38: 753-8.

[5] Darmaun D, Smith SD, Sweeten S, Sager BK, Welch S, Mauras N. Evidence for accelerated rates of glutathione utilization and glutathione depletion in adolescents with poorly controlled type 1 diabetes. Diabetes 2005;54:190-6.

[6] Lyons J, Rauh-Pfeiffer A, Ming-Yu Y, Lu XM, 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 2001;29:870-7.

[7] Franco R, Schoneveld OJ, Pappa A, Panayiotidis MI. The central role of glutathione in the pathophysiology of human diseases. Arch Physiol Biochem 2007;113:234-58.

[8] 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 2002;76: 646-52.

[9] De Rosa SC, Zaretsky MD, Dubs JG, Roederer M, Anderson M, Green A, Mitra D, Watanabe N, Nakamura H, Tjioe I, Deresinski SC, Moore WA, Ela SW, Parks D, Herzenberg LA, Herzenberg LA. *N*-acetylcysteine replenishes glutathione in HIV infection. Eur J Clin Invest 2000;30:915-29.

[10] Jahoor F, Jackson A, Gazzard B, Philips G, Sharpstone D, Frazer ME, Heird W. Erythrocyte glutathione deficiency in symptom-free HIV infection is associated with decreased synthesis rate. Am J Physiol 1999;276:E205-11.

[11] Biolo G, Antonione R, De Cicco M. Glutathione metabolism in sepsis. Crit Care Med 2007;35:S591-5.

[12] Mizock BA. Alterations in carbohydrate metabolism during stress: a review of the literature. Am J Med 1995;98:75-84.

[13] Diagnosis and classification of diabetes mellitus. Diabetes Care 2008; 31(Suppl 1):S55–S60.

[14] Stegenga ME, van der Crabben SN, Levi M, de Vos AF, Tanck MW, Sauerwein HP, van der Poll T. Hyperglycemia stimulates coagulation, whereas hyperinsulinemia impairs fibrinolysis in healthy humans. Diabetes 2006;55:1807-12.

[15] Stegenga ME, van der Crabben SN, Dessing MC, Pater JM, van den Pangaart PS, de Vos AF, Tanck MW, Roos D, Sauerwein HP, van der Poll T. Effect of acute hyperglycaemia and/or hyperinsulinaemia on proinflammatory gene expression, cytokine production and neutrophil function in humans. Diabet Med 2008;25:157-64.

[16] Stegenga ME, van der Crabben SN, Blumer RM, Levi M, Meijers JC, Serlie MJ, Tanck MW, Sauerwein HP, van der Poll T. Hyperglycemia enhances coagulation and reduces neutrophil degranulation, whereas hyperinsulinemia inhibits fibrinolysis during human endotoxemia. Blood 2008;112:82-9.

[17] Blumer RM, van der Crabben SN, Stegenga ME, Tanck MW, Ackermans MT, Endert E, van der Poll T, Sauerwein HP. Hyperglycemia prevents the suppressive effect of hyperinsulinemia on plasma adiponectin levels in healthy humans. Am J Physiol Endocrinol Metab 2008;295:E613-7.

[18] van der Crabben SN, Blumer RM, Stegenga ME, Ackermans MT, Endert E, Tanck MW, Serlie MJ, van der Poll T, Sauerwein HP. Early endotoxemia increases peripheral and hepatic insulin sensitivity in healthy humans. J Clin Endocrinol Metab 2009;94:463-8.

[19] Soeters MR, Sauerwein HP, Groener JE, Aerts JM, Ackermans MT, Glatz JF, Fliers E, Serlie MJ. Gender-related differences in the metabolic response to fasting. J Clin Endocrinol Metab 2007;92: 3646-52.

[20] Capitan P, Malmezat T, Breuille D, Obled C. Gas chromatographic–mass spectrometric analysis of stable isotopes of cysteine and glutathione in biological samples. J Chromatogr B Biomed Sci Appl 1999;732:127-35.

[21] Humbert B, Nguyen P, Obled C, Bobin C, Vaslin A, Sweeten S, Darmaun D. Use of L-[(15)N] glutamic acid and homoglutathione to determine both glutathione synthesis and concentration by gas chromatography–mass spectrometry (GCMS). J Mass Spectrom 2001;36:726-35.

[22] Travis SF, Morrison AD, Clements Jr RS, Winegrad AI, Oski FA. Metabolic alterations in the human erythrocyte produced by increases in glucose concentration. The role of the polyol pathway. J Clin Invest 1971;50:2104-12.

[23] Blanco RA, Ziegler TR, Carlson BA, Cheng PY, Park Y, Cotsonis GA, Accardi CJ, Jones DP. Diurnal variation in glutathione and cysteine redox states in human plasma. Am J Clin Nutr 2007;86:1016-23.
[24] Jahoor F, Wykes LJ, Reeds PJ, Henry JF, del Rosario MP, Frazer ME. Protein-deficient pigs cannot maintain reduced glutathione homeostasis when subjected to the stress of inflammation. J Nutr 1995;125: 1462-72.
[25] Malmezat T, Breuille D, Capitan P, Mirand PP, Obled C. Glutathione turnover is increased during the acute phase of sepsis in rats. J Nutr 2000;130:1239-46.
[26] Lowry SF. Human endotoxemia: a model for mechanistic insight and therapeutic targeting. Shock 2005;24(Suppl 1):94-100.
[27] van der Crabben SN, Wijburg FA, Ackermans MT, Sauerwein HP. Effect of cysteine dosage on erythrocyte glutathione synthesis in a patient with cystathionine beta synthase deficiency. J Inherit Metab Disease 2008.
[28] van der Crabben SN, Allick G, Ackermans MT, Endert E, Romijn JA, Sauerwein HP. Prolonged fasting induces peripheral insulin resistance which is not ameliorated by high-dose salicylate. J Clin Endocrinol Metab 2008;23:638-41.