

## Rise in Plasma Oxidized Glutathione by Experimental Hypoglycemia

ZHONG-LI JIANG and TOKUTARO SATO

*The Section of Internal Medicine and Disability Prevention,  
Disability Science, Division of Medicine, Tohoku University  
Graduate School, Sendai 980-8574*

JIANG, Z.-L. and SATO, T. *Rise in Plasma Oxidized Glutathione in Experimental Hypoglycemia.* Tohoku J. Exp. Med., 1999, 187 (1), 59-64 — Changes in plasma glutathione were investigated under hypoglycemic status. Twelve rabbits were randomly divided into hypoglycemic group ( $n=6$ ) and saline-injected control group ( $n=6$ ). Hypoglycemia was induced by intravenous injection of insulin as 10 U/kg and recovered by intravenous glucose injection after 60 minutes. In the control group, saline was intravenously injected in stead of insulin. Plasma levels of oxidized glutathione (GSSG) rose significantly ( $p<0.01$ ) and remarkably decrease in plasma GSH/GSSG ratio ( $p<0.05$ ) accompanying increase in serum enzymes in the hypoglycemic group. These results suggest that hypoglycemia might cause change in plasma GSSG which is related to increase of serum enzymes by hypoglycemia. ——— hypoglycemia; reduced glutathione (GSH); oxidized glutathione (GSSG) © 1999 Tohoku University Medical Press

The alteration of plasma levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) has been demonstrated in many physiological and pathological conditions as fasting, exercise, hepatic damage and ischemia-reperfusion damage (Martensson 1986; Galinanes et al. 1992; Ji and Ronggen 1992; Denno et al. 1995). As hypoglycemia is similar changes in energy metabolism and microcirculation to ischemia-reperfusion damage, it is unknown whether hypoglycemia may also contribute to oxidant status in blood. We reported that insulin-induced hypoglycemia raised plasma activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK) in rabbit, which suggested damage in muscle rather than in liver (Jiang et al. 1996). In our present experiment, we studied on changes of plasma glutathione, in order to explicate mechanism of muscle damage caused by hypoglycemia.

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Address for reprints: Zhong-Li Jiang, M.D., The Section of Internal Medicine and Disability Prevention, Disability Science, Division of Medicine, Tohoku University Graduate School, Sendai 980-8574, Japan.

e-mail: zhongli@mail.cc.tohoku.ac.jp

## MATERIALS AND METHODS

Twelve male Japanese white rabbits (age 20 weeks, body weight 2.6~3.0 kg) were obtained from Funabashi Farm (Chiba) and maintained on a regular diet (RC-4 Oriental Yeast Co., Chiba). The rabbits were randomly divided into hypoglycemic group (H group,  $n=6$ ) and saline-injected control group (C group,  $n=6$ ). All experiments were performed on 6 hours from 9:00 a.m. to 3:00 p.m. Acute hypoglycemia with 60 minute duration was induced by an intravenous injection of insulin (Novolin R 40, Novo Nordisk, Copenhagen, Denmark) in a dosage of 10 U/kg and recovered by intravenous injection of 5 ml of 50% glucose solution at 60, 90, 120 and 150 minutes after insulin injection (Jiang et al. 1998). As a placebo, 1 ml of saline was intravenously injected at 0 minute and 5 ml at 60, 90, 120, 150 minutes in C group. All rabbits were fasted for one hour after insulin or saline injection.

Blood glucose concentrations were determined before and at 30, 60, 90, 120, 150, 240, and 360 minutes after insulin or saline injection on Drichem System (Fuji, Tokyo). For glutathione and potassium analysis, 0.8 ml of blood samples was taken anaerobically before and at 6, 24 and 48 hours after the insulin or saline injection. The ethylenediaminetetraacetic acid (EDTA)-treated blood was centrifuged in a microfuge (Beckman, 10 000 g) for 1.5 minutes. The plasma was deproteinized with 5-sulfosalicylic acid (10%, w/v) and again centrifuged for 5 minutes. Plasma potassium was assayed on an EA03 automated electrolyte analyzer (A & T, Tokyo).

Plasma levels of GSH and GSSG were measured according to the method of DTNB [5, 5'-Dithiobis (2-nitrobenzoic Acid)]-GSSG reductase recycling assay as Anderson (1985) reported. For measurement of GSH, 25  $\mu$ l of the supernatant was added to 700  $\mu$ l of daily buffer, 100  $\mu$ l of 6 mM DTNB and 175  $\mu$ l of water, and warmed at 30°C in a water bath for 15 minutes after mixing. 10  $\mu$ l of glutathione reductase (Sigma, St. Louis, MO, USA) was added with mixing to initiate the assay. The rate of 5-thio-2-nitrobenzoic acid (TNB) formation was followed at 412 nm on a spectrophotometer (U2001, Hitachi, Tokyo). The amount of GSH was determined from a standard curve made with GSH (Sigma, St. Louis, MO, USA) solutions of 0.1, 0.2, 0.5 and 1 nM/25  $\mu$ l.

For GSSG determination, the modification of this procedure described by Griffith (1980) was utilized, in which 2-vinylpyridine is used to mask the sulfhydryl group of GSH. The 5-sulfosalicylic acid supernatant solution (100  $\mu$ l) was mixed with 2  $\mu$ l of 2-vinylpyridine and pH was adjusted between 6 and 7 with triethanolamine. After 60 minutes, the derivatized samples were assayed as described above.

All data are expressed as the mean  $\pm$  s.d. Data between H and C groups were analyzed using analysis of variance (ANOVA). The changes of plasma glutathione before and after insulin injection in the same group were analyzed with

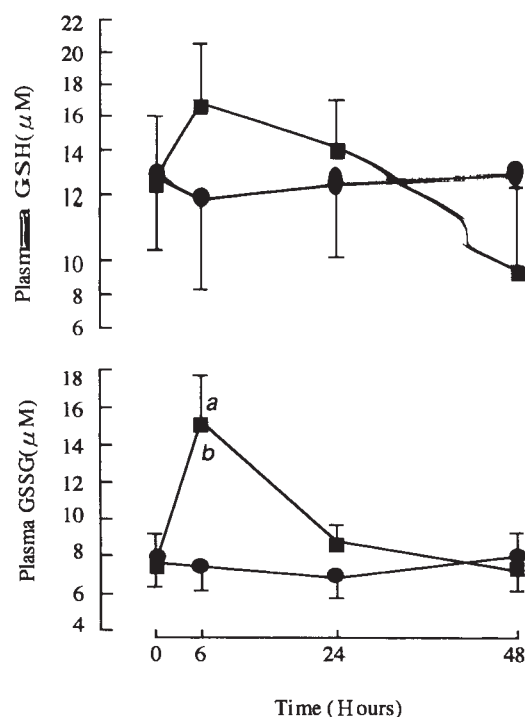


Fig. 1. Changes of plasma GSH and GSSG in hypoglycemic group (■) and control group (●). <sup>a</sup> $p < 0.01$  vs. initial values; <sup>b</sup> $p < 0.01$  vs. control group.

paired *t*-test. Level of significance was defined as  $p < 0.05$ .

## RESULTS

The blood glucose levels decreased significantly from 104 mg/100 ml at baseline to the levels of 55 and 49 mg/100 ml at 30 and 60 minutes, respectively, after the insulin injection, and recovered to 163, 184, 203, 112, 118 mg/100 ml at 90, 120, 150, 240, 360 minutes, respectively, by intravenous glucose injection in H group. In C group, the blood glucose levels were 110, 100, 107, 97, 107, 112, 107, 115 mg/ml at 0, 30, 60, 90, 120, 150, 240, 360 minutes, respectively, during the experiment.

Plasma potassium showed no significant changes during the experiment in both groups, which excluded the possibility of changes in GSH due to hemolysis.

Fig. 1 shows the changes in plasma GSH and GSSG in H and C groups. Plasma GSH tended to increase at 6 hours after insulin injection in H group ( $p = 0.07$ ). A remarkable rise in plasma GSSG ( $p < 0.01$ ) and a significant decrease in plasma GSH/GSSG ratio from 1.68 to 1.15 ( $p < 0.05$ ) were observed at 6 hours in H group. In C group, any influential changes were not found during the experiment.

The changes in serum enzymes in H and C groups are shown in Table 1. In H group, serum CK, ALT, AST and LDH activities increased significantly at 6 hours after insulin injection, in which serum CK was significantly higher than that in C group at 6 hours after insulin or saline injection. However, no remarkable changes in serum enzyme activities were observed in C group.

TABLE 1. *Changes in serum enzymes by experimental hypoglycemia*

Enzyme	Group ( $n=6$ )	Initial	6 hours*
CK (U/L)	H	320 $\pm$ 39	1006 $\pm$ 434 <sup>a,c</sup>
	C	262 $\pm$ 78	303 $\pm$ 105
ALT (U/L)	H	57 $\pm$ 20	62 $\pm$ 19 <sup>a</sup>
	C	46 $\pm$ 13	55 $\pm$ 25
AST (U/L)	H	28 $\pm$ 8	50 $\pm$ 14 <sup>b</sup>
	C	22 $\pm$ 6	34 $\pm$ 23
LDH (U/L)	H	116 $\pm$ 25	205 $\pm$ 31 <sup>b</sup>
	C	122 $\pm$ 21	147 $\pm$ 17

H, hypoglycemic group; C, control group. Values are means $\pm$ s.d.

\*Six hours after injection of insulin or saline.

<sup>a</sup> $p < 0.05$  vs. initial values by paired  $t$ -tests.

<sup>b</sup> $p < 0.01$  vs. initial values by paired  $t$ -tests.

<sup>c</sup> $p < 0.01$  vs. values at 6 hours in control group by Student's  $t$ -tests.

## DISCUSSION

Glutathione, an antioxidant, is a physiological constituent of plasma. Under normal conditions, the balance of equation is far in the direction of maintaining cellular glutathione in its reduced form. Conversion of GSH to GSSG by oxidation results in a considerable loss of the former because of an efflux in tissue (Lu et al. 1990). This efflux is well demonstrated in cells exposed to experimentally-induced oxidative stress (Deneke and Fanburg 1989). Our results demonstrated that insulin-induced hypoglycemia may increase plasma GSSG and decrease plasma GSH/GSSG ratio, leading oxidant status in plasma.

The plasma levels of glutathione are reported to rise in hepatic damage (Denno et al. 1995) and ischemia-reperfusion injury of myocardium (Galinanes et al. 1992). In our preliminary study, we reported the increase of serum enzymes by hypoglycemia (Jiang et al. 1996). Recently, we also showed that the increase in both serum enzyme and CK-isoenzyme activities during hypoglycemia is primarily due to damage in heart and skeletal muscles rather than liver, and that the duration of hypoglycemia and insulin dosage may influence the extent of organ damage (Jiang et al. 1998). In the present study, we observed that plasma GSSG increased significantly and plasma GSH/GSSG ratio reduced significantly at 6 hours after insulin injection in H group. These changes were consistent with the increase of serum enzymes displayed in our present study. It may suggest that insulin-induced hypoglycemia is related to oxidative damage of tissue represented increase in serum enzymes. Further study is necessary to elucidate mechanisms of elevation of serum enzymes and of changes in plasma glutathione

by hypoglycemia.

During recovery period blood glucose levels at 90~150 minutes remarkably exceeded initial values in H group. In our previous study, we observed similar hyperglycemic status in control group with insulin plus multiple glucose injections, in which serum enzyme activities did not increase significantly at 6 hours after injection (Jiang et al. 1996). And then, we also evidenced that the increase in serum enzymes was mainly influenced by hypoglycemic duration (Jiang et al. 1998). In addition, we found that plasma GSH/GSSG ratio varied from  $1.77 \pm 0.36$  to  $1.80 \pm 0.43$  at 0 and 6 hours respectively in the other control group with "rebound hyperglycemia" injected by insulin plus multiple glucose, showing no significant difference. Therefore, rebound hyperglycemia itself could not reduce plasma GSH/GSSG ratio.

GSH in plasma rapidly undergoes auto-oxidation in vitro to GSSG and other oxidation products (Anderson 1985). In the present study, plasma GSH and GSSG values are in agreement with the finding reported by Costagliola et al. (1987). We also compared two different methods with N-ethylmaleimide (NEM) or 2-vinylpyridine to treat samples, and demonstrated that either NEM or 2-vinylpyridine could sufficiently prevent GSH to be oxidized to GSSG, which is in good agreement with the results reported by Griffith (1980).

In summary, this study demonstrates that insulin-induced hypoglycemia significantly increased plasma GSSG and declined plasma GSH/GSSG ratio, accompanying increase in serum enzymes. The results suggest that change in plasma GSSG induced by hypoglycemia is related to increase of serum enzymes.

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