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Role of glutathione in an animal model of myoglobinuric acute renal failure

SAMEH R. ABUL-EZZ, PATRICK D. WALKER, AND SUDHIR V. SHAH*

Tulane University School of Medicine, Departments of Medicine and Pathology, New Orleans, LA 70112

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ABSTRACT **In a previous study we have shown a role for reactive oxygen metabolites in glycerol-induced acute renal failure, a well-established model for myoglobinuric acute renal failure. In the present study we examined the role of glutathione in this model of acute renal failure. Administration of 50% (vol/vol) glycerol at a dose of 10 ml/kg of body weight to rats intramuscularly resulted in significant renal failure associated with depletion of total kidney glutathione (GSH) from 2.6 ± 0.1 μmol/g (mean ± SEM control level) to 1.7 ± 0.1 μmol/g after 6 hr ($P < 0.001$). If GSH were important in glycerol-induced acute renal failure, one would anticipate that exogenously administered GSH should afford protection, while injury should be potentiated if endogenous GSH is depleted. We examined the effect of i.p. administration of L-buthionine-(*S,R*)-sulfoximine (BSO) at 2 mmol/kg (which results in depletion of kidney GSH) and the effect of increasing renal GSH by i.v. administration of reduced GSH (2 mmol/kg every 3 hr) on kidney function in glycerol-treated rats. Glycerol-injected rats treated with BSO showed significantly worse renal failure than did rats given glycerol alone, while administration of GSH resulted in significant amelioration of glycerol-induced acute renal failure [glycerol treatment alone, blood urea nitrogen (BUN) = 96 ± 10 and creatinine = 2.5 ± 0.4 mg/dl; BSO + glycerol treatment, BUN = 123 ± 7 and creatinine = 3.5 ± 0.1 mg/dl ($n = 9$, $P < 0.05$); GSH + glycerol treatment, BUN = 78 ± 10 and creatinine = 1.25 ± 0.2 mg/dl ($n = 8$, $P < 0.05$)]. In separate experiments 1,3-bis(chloroethyl)-1-nitrosourea (BCNU) [which interferes with the enzyme GSH reductase and prevents recycling of oxidized GSH (GSSG) into GSH] resulted in worsening of glycerol-induced acute renal failure similar to that produced by BSO. These functional differences between GSH-depleted and GSH-repleted rats were further substantiated by significant histological differences in tubular injury. Taken together, these results provide evidence for an important role of GSH in glycerol-induced acute renal failure.**

During the Battle of Britain, Bywaters and Beall (1) described the first causative association of acute renal failure with skeletal muscle injury and the release of muscle cell contents, including myoglobin, into plasma (rhabdomyolysis). Since then the spectrum of etiologies for rhabdomyolysis, myoglobinuria, and renal failure has been markedly expanded, with both traumatic and, more recently, nontraumatic causes being recognized (2–5). Rhabdomyolysis may also occur in apparently normal subjects after prolonged strenuous exercise including marathon runners, ice skaters, and football players after competition and in as many as 39% of the Marine Corps recruit population (6). It is estimated that about one-third of the patients with rhabdomyolysis will develop acute renal failure (2) and recent studies show that it is not an infrequent cause of renal failure accounting for 5–7% of all cases of acute renal failure (3).

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The most widely applied model of myoglobinuric acute renal failure is produced by s.c. or i.m. injection of hypertonic glycerol (7). Recently, a role for reactive oxygen metabolites in glycerol-induced acute renal failure has been suggested based on the protective effect of hydroxyl radical scavengers (8), and deferoxamine (8, 9) (which chelates iron that participates in hydroxyl radical formation). In addition, *in vivo* enhanced generation of hydrogen peroxide by the renal cortex has been reported in the same model (10).

Glutathione (GSH), a tripeptide, occurs in high concentrations in virtually all mammalian cells and is the most prevalent intracellular thiol (11–13). GSH has many diverse functions, one of which is the protection against oxidative damage. The importance of GSH in protecting cells against oxidant injury has been delineated in numerous *in vitro* studies where depletion of GSH resulted in markedly enhanced toxicity (14–20) and increased nonprotein sulfhydryl content provided protection (14, 21–24). Although the role of GSH in *in vivo* studies has not been examined as extensively as in studies *in vitro*, its role has been suggested in several disease states, including ischemia/reperfusion injury to the heart (25, 26), adriamycin toxicity (21), hepatotoxicity (27), and in L-buthionine-(*S,R*)-sulfoximine (BSO)-induced cataracts (28).

Several features (29, 30), including the rapid GSH turnover in the kidney (31) and the ability of the kidney to effectively clear the circulation of GSH (32), distinguish the GSH metabolism in the kidney from other organs. In accord with studies in other tissues, *in vitro* studies have shown the protective effect of GSH in oxidant injury to the proximal renal tubules (33–35). However, the role of GSH in *in vivo* models of renal injury in which reactive oxygen metabolites have been postulated to play an important role (36, 37) remains controversial. Thus, Paller (38) reported a protective effect of intravenous GSH in ischemic acute renal failure, whereas Zager (39) failed to find a protective effect. Scaduto *et al.* (40) reported that prior depletion of GSH with BSO did not enhance and that GSH monoethyl ester did not protect in ischemic acute renal failure.

The purpose of this study was to examine the role of GSH in glycerol-induced acute renal failure. We first examined the effect of glycerol-induced acute renal failure on kidney GSH. We then examined the effect of exogenously administered GSH, and the effect of depleting kidney GSH on glycerol-induced acute renal failure. If GSH were important in glycerol-induced acute renal failure, one would anticipate that exogenously administered GSH should afford protection, whereas injury should be potentiated if endogenous glutathione were depleted.

METHODS

Animal Model. Adult male Sprague–Dawley rats (250–350 g) were used in all of the experiments. Renal failure was

Abbreviations: GSH, glutathione; GSSG, oxidized glutathione; BUN, blood urea nitrogen; BSO, L-buthionine-(*S,R*)-sulfoximine; BCNU, 1,3-bis(chloroethyl)-1-nitrosourea.

*To whom reprint requests should be sent at the present address: Division of Nephrology, University of Arkansas for Medical Sciences, 4301 W. Markham, Slot 501, Little Rock, AR 72205.

produced by rats deprived of water for 24 hr followed by i.m. injection with 50% (vol/vol) glycerol (10 ml/kg of body weight) as described in our previous studies (8). Rats were sacrificed 16 hr later (by use of sodium pentobarbital at 50 mg/kg administered i.p., followed by exsanguination); blood was obtained for blood urea nitrogen (BUN), creatinine, and aldolase assays, and kidney tissue was used for determination of GSH/GSSG (oxidized GSH) levels and for histology.

The effect of glycerol-induced acute renal failure on kidney GSH/GSSG content. To study the effect of glycerol-induced acute renal failure on kidney GSH/GSSG content, three groups of rats (six in each group) were studied. These groups were sacrificed at 1, 6, and 16 hr after glycerol injection, and the fourth group (n = 5) served as control.

The effect of depleting or increasing kidney GSH levels on glycerol-induced acute renal failure. To deplete kidney GSH, BSO was administered i.p. at a dosage of 2 mmol/kg every 12 hr (total of two doses) with the first dose administered 4 hr before glycerol injection. To inhibit reduction of GSSG into GSH, BCNU was administered i.p. at a dosage of 80 mg/kg 12 hr before glycerol injection. Increasing kidney GSH level was accomplished by injecting GSH at a dosage of 2 mmol/kg (in a volume of 0.5 ml/kg) intravenously every 3 hr for a total of five doses. The first dose was administered simultaneously with the glycerol injection.

The effect of depleting or increasing kidney GSH levels on glycerol-induced acute renal failure was studied in four groups of rats (eight or nine rats in each group). Group I received i.m. 10 ml of 0.9% NaCl per kg after 24 hr without water. Group II received i.m. glycerol, and groups III and IV received either BSO i.p. or GSH i.v. in addition to i.m. glycerol. To adjust for the additional volume administered to groups III and IV, an appropriate amount of normal saline was administered to group II rats. Two additional groups of rats (n = four in each group) were administered either BSO or GSH alone. In a separate experiment, the effect of BCNU and BSO on glycerol-induced acute renal failure was examined. Rats were divided into four groups (four to six in each group). Group I received intramuscular saline, group II received intramuscular glycerol, while groups III and IV received BSO and BCNU, respectively, in addition to intramuscular glycerol.

Assays. *Kidney GSH and GSSG determination.* Freeze-clamped kidney was crushed at liquid nitrogen temperature. Total GSH was measured spectrophotometrically by the recirculating enzymatic assay described by Tietze (41). Masking of GSH with 2-vinylpyridine was utilized to measure GSSG (42). Tissue deproteinization was achieved with perchloric acid (1:5, wt/vol). After centrifugation, the pH of the supernatant was adjusted by adding 2 M KOH in 0.3 M Mops. In some experiments, a weighed portion of freeze-clamped kidneys was dried at 80°C for 72 hr. The ratio of wet to dry weight did not change significantly between groups, so the results were expressed as μmol/g wet weight.

BUN and serum creatinine were determined by using the Beckman BUN analyzer 2 and the Beckman Creatinine analyzer. Serum aldolase was measured colorimetrically with modifications by using Sigma Diagnostic Kit 752.

Kidney histology. Kidneys were taken for histology from some of the rats in the experiments described above. The kidneys were sectioned, and a portion was fixed in 10% formalin, dehydrated, and embedded in Paraplast. Sections were cut at 3 μm and stained with periodic acid–Schiff base reagent. The slides were coded, and a semiquantitative analysis of the kidney sections was performed without knowledge of the treatment protocol on two separate occasions as in our previous study (8). The changes seen were limited to the tubulointerstitial areas and consisted of vacuolar degeneration and epithelial cell swelling (referred to as vacuoles) and epithelial cell necrosis and desquamation (referred to as necrosis). The degree of change was graded as follows: 0, normal; trace, changes involving <5% of cortical tubules; 1, changes involving >5% but <25% of cortical tubules; 2, changes involving >25% but <50% of cortical tubules; 3, changes involving >50% but <75% of cortical tubules; 4, changes involving >75% of cortical tubules (8).

Reagents. BSO was obtained from Chemical Dynamics, and BCNU was from Bristol–Myers Squibb (Syracuse, NY). GSH, GSSG, GSH reductase, NADPH, and other miscellaneous reagents were obtained from Sigma.

Statistical analysis. All measured values are reported as mean ± SEM. For normally distributed data, the groups were compared by using analysis of variance and were compared post hoc with the Fischer least significant difference (LSD) procedure. Statistical significance was considered at P values < 0.05.

RESULTS

The effect of i.m. injection of glycerol on total renal GSH is shown in Fig. 1. As expected, there was a marked increase in BUN and serum creatinine over time after glycerol injection (Fig. 1 *Lower*). Fig. 1 *Upper* shows the change in renal GSH and GSSG measured at the same time points as BUN and serum creatinine. There was marked depletion of renal

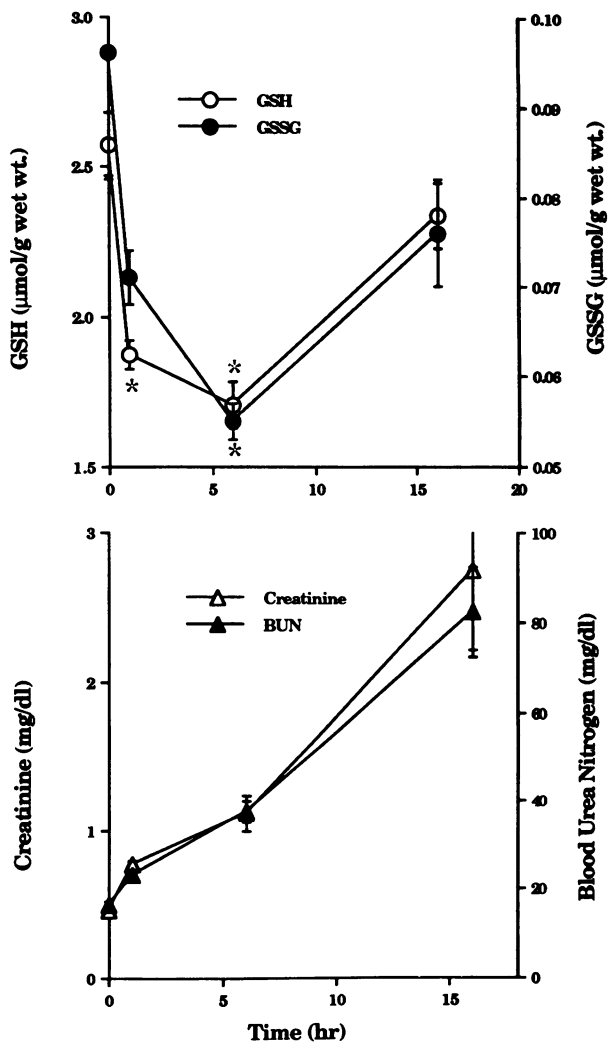

FIG. 1. Effect of glycerol-induced acute renal failure on total kidney GSH and GSSG. Results (means ± SEM) are from three groups of rats (n = 6) in each group, sacrificed at 1, 6, and 16 hr after injection i.m. of 50% glycerol (10 ml/kg). Rats injected with 0.9% saline (n = 5) served as controls at time 0. *, $P < 0.001$; wt., weight.

GSH early after glycerol injection to 73 ± 2% of the control value by 1 hr, and the decrease continued to 66 ± 3% of the control value by 6 hr. Sixteen hours after glycerol injection, GSH returned almost to the baseline level (91 ± 4%). Since similar changes were observed in renal GSSG, there were no significant changes in the GSH redox ratio, which remained around 3%.

In preliminary studies, we examined the effect of BSO and GSH injections on renal GSH levels. BSO at a dose of 2 mmol/kg injected i.p. produced in 4 hr an 86 ± 1% depletion of renal GSH that lasted for 12 hr before the GSH level began to increase. In addition, intravenous injection of GSH (2 mmol/kg) produced a significant increase in renal GSH (69 ± 18%) in 2 hr, returning to baseline in 4 hr.

We then examined the effect of increasing or decreasing renal GSH in glycerol-induced acute renal failure (Fig. 2). Two groups of rats were pretreated with either BSO or GSH before glycerol injection, with the maintenance dosage as detailed in methods. Two other groups of rats received either glycerol or saline intramuscularly. As expected, rats injected with intramuscular glycerol had significantly higher BUN and creatinine levels compared with the saline controls. Rats that received BSO to deplete GSH had significantly higher BUN and creatinine levels compared with rats receiving either glycerol alone or glycerol and GSH. Rats that received GSH in addition to glycerol had significantly lower BUN and creatinine levels compared with those receiving glycerol alone or glycerol and BSO.

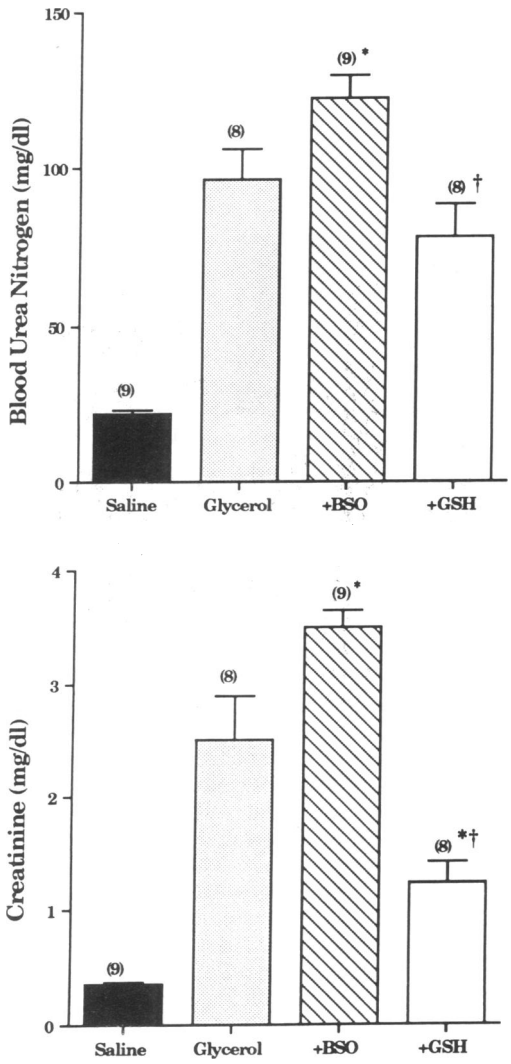

FIG. 2. Effect of depleting or increasing kidney GSH content on glycerol-induced acute renal failure as measured by BUN (*Upper*) and serum creatinine (*Lower*). Results are means ± SEM for each group 16 hr after injection of 50% glycerol (10 ml/kg) or 0.9% saline (for the control group). *, Significantly different from the group treated with glycerol alone ($P < 0.05$); †, significantly different from the glycerol/BSO-treated group ($P < 0.001$); *†, significantly different from both of the other groups.

Table 1. Semiquantitative analysis of renal histology

	Vacuoles						Necrosis					
	0	Trace	1+	2+	3+	4+	0	Trace	1+	2+	3+	4+
Control	4	—	—	—	—	—	4	—	—	—	—	—
Glycerol	—	—	—	1	2	1	2	—	1	1	—	—
Glycerol/GSH	—	2	1	1	—	—	2	1	1	—	—	—
Glycerol/BSO	—	—	—	—	3	1	—	—	1	2	1	—

Histological grading is as follows: vacuoles = vacuolar degeneration and epithelial cell swelling; necrosis = necrosis and epithelial desquamation. 0, normal; trace, changes involving <5% of cortical tubules; 1, changes involving >5% but <25% of cortical tubules; 2, changes involving >25% but <50% of cortical tubules; 3, changes involving >50% but <75% of cortical tubules; 4, changes involving >75% of cortical tubules. GSH, reduced glutathione; BSO, L-buthionine-SR-sulfoximine. Each number represents the findings from one animal (total of four rats in each group, each rat evaluated for vacuoles and necrosis).

These functional differences between the GSH and BSO groups were also confirmed histologically (Table 1). Semiquantitative analysis of the histological changes revealed a moderate degree of tubular damage in the rats treated with glycerol alone. Glycerol-injected rats treated with GSH showed much less histological evidence of renal injury, while those glycerol-injected rats receiving BSO demonstrated greater renal vacuolization and necrosis (Fig. 3).

These differences were not due to interference of these agents with the degree of muscle injury. Serum aldolase, a measure of muscle damage, was determined in the four experimental groups, and no differences were found in the groups receiving glycerol with or without BSO or GSH [saline controls, 12.2 ± 0.4 units/ml; glycerol treatment, 23.8 ± 0.9 units/ml; glycerol/BSO treatment, 22.8 ± 0.9 units/ml; glycerol/GSH treatment, 21.7 ± 1.1 units/ml (n = 6–9)]. These differences in renal function and histology could not be attributed to a direct effect of these compounds on the kidney. No significant change in renal function (as measured by BUN and serum creatinine levels) was observed after administration of BSO or GSH alone [BSO treatment alone (n = 3), BUN 20 ± 1 mg/dl and creatinine 0.3 ± 0.1 mg/dl; GSH treatment alone (n = 3), BUN 15 ± 1 mg/dl and creatinine 0.3 ± 0.1 mg/dl (P = not significant)].

Using a similar experimental design, we determined the effect of pretreatment with BCNU (which interferes with the enzyme GSH reductase, preventing recycling of GSSG into GSH). As in the previous experiments, rats in which GSH was depleted with BSO had significantly worse renal function as measured by BUN and serum creatinine levels (Fig. 4). In addition, rats pretreated with BCNU also had higher creatinine levels compared with the group treated with the glycerol only (Fig. 4).

DISCUSSION

Injection i.m. of glycerol, an animal model of rhabdomyolysis, produced significant and early depletion of renal GSH. Similar renal GSH depletion has been reported in other models of renal injury such as ischemia/reperfusion injury (40, 43) and direct exposure to reactive oxygen metabolites (44).

We did not demonstrate any significant increase in renal GSSG at any time point after glycerol injection. Depletion of

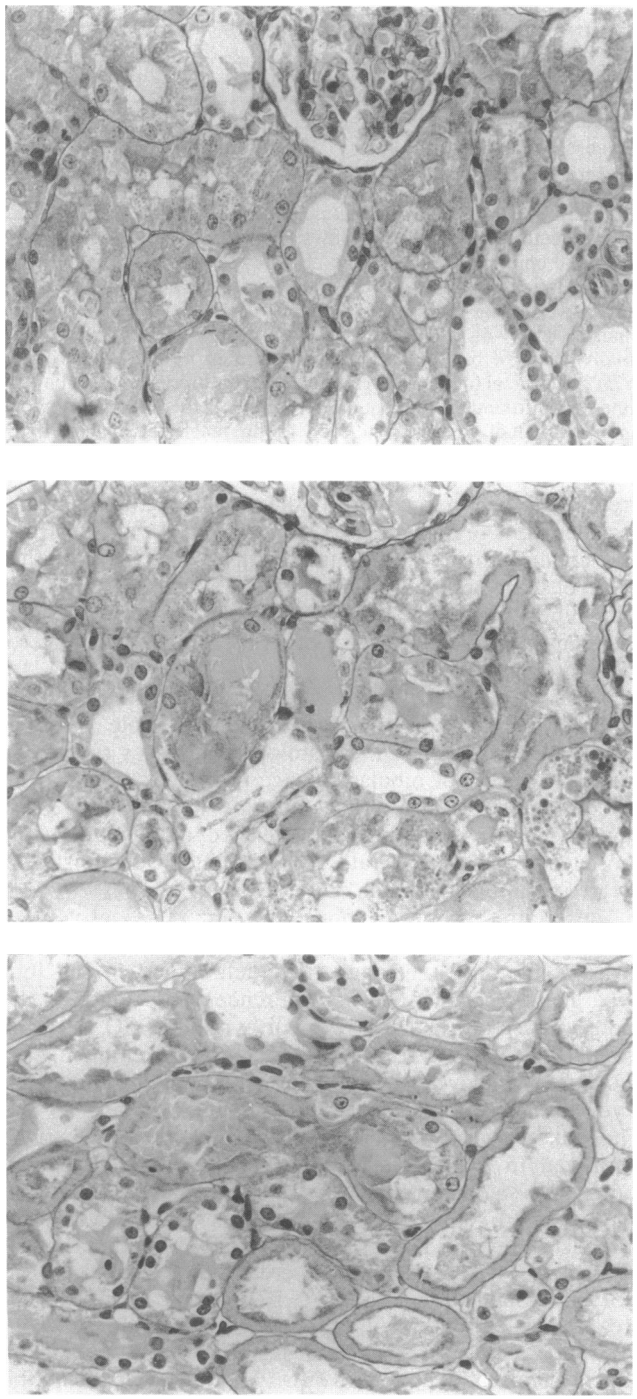

FIG. 3. (*Top*) Light-microscopic section of kidney from rat receiving glycerol and GSH, showing moderate epithelial cell vacuolar change and swelling with a small focus of necrosis (vacuoles, grade 2; necrosis, grade 1). (*Middle*) Light-microscopic section of kidney from rat receiving glycerol alone, showing moderate to severe epithelial cell vacuolar change and swelling with more necrosis (vacuoles, grade 3; necrosis, grade 2). (*Bottom*) Light-microscopic section of kidney from rat receiving glycerol and BSO, showing almost complete tubular cell necrosis, with the residual cells showing extensive epithelial cell vacuolar change and swelling (vacuoles, grade 3; necrosis, grade 3). [Periodic acid–Schiff base; ×200 (all panels).]

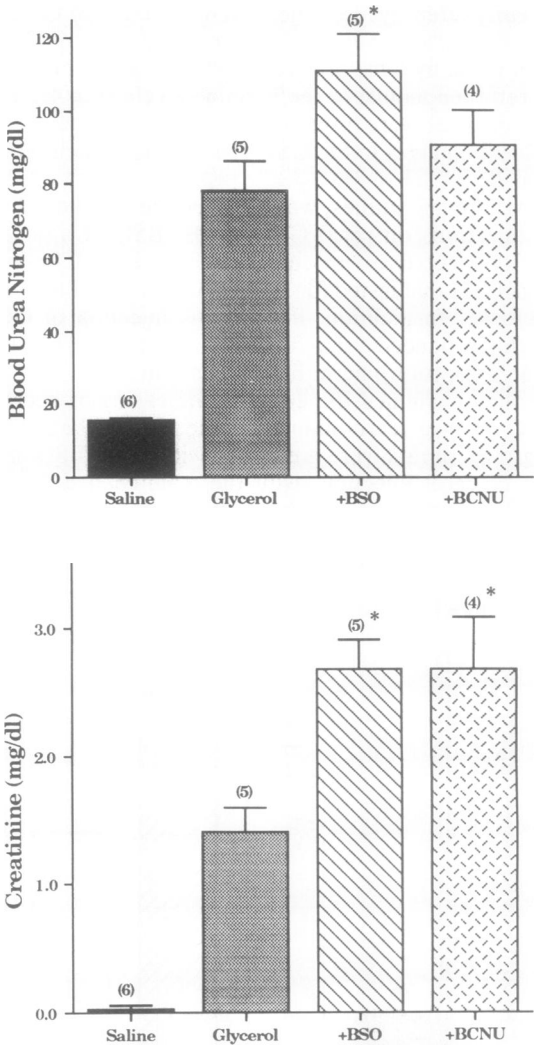

FIG. 4. Effect of BSO and BCNU on glycerol-induced acute renal failure as measured by BUN (*Upper*) and serum creatinine (*Lower*). Results are means ± SEM for each group 16 hr after injection of 50% glycerol (10 ml/kg) or 0.9% saline (for the control group). *, Significantly different from the group treated with glycerol alone ($P < 0.05$).

renal GSSG paralleled that of GSH, maintaining the redox ratio at baseline level throughout the experiment. An increase in the GSH redox ratio has been demonstrated by McCoy *et al.* following renal ischemia (43). Other investigators did not demonstrate such an increase in the redox ratio after renal ischemia and attributed the decrease in total kidney GSH to the lack of synthesis of GSH secondary to ATP depletion in that model (40). An alternative explanation is the oxidation of GSH to GSSG by the oxidant stress, with efflux of GSSG being the major factor responsible for maintenance of the redox ratio. Such an efflux has been shown in various hepatic models, including *in vivo* biliary efflux of GSSG in rats (45). Earlier studies have demonstrated unidirectional GSSG efflux from human erythrocytes when exposed to hydrogen peroxide, despite a large concentration gradient (46, 47).

The role of GSH was delineated by examining the effect of increasing or decreasing renal GSH in glycerol-induced acute renal failure. Intravenous injection of GSH resulted in significant functional protection as measured by BUN and creatinine levels and by assessment of the renal tissue histologically. Much of the current evidence favors the concept that GSH is broken down to its constituent amino acids or dipeptides, which are then transported in cells where intracellular enzymes catalyze synthesis of GSH (48, 49). In addition, administration of GSH is associated with glutathionuria (50). Thus, the protective effect of GSH may be due to either increased intracellular GSH levels or the presence of GSH in tubular fluid, or both. Additional support for the role of GSH is derived from the observation that in glycerol-

treated rats, GSH-depleted rats had a significantly worse renal function and tissue injury as assessed histologically compared with rats that received GSH or rats that received glycerol alone. These differences were not due to interference of BSO or GSH with the degree of muscle injury.

In separate experiments, BCNU resulted in worsening of glycerol-induced acute renal failure similar to that produced by BSO. BSO interferes with GSH synthesis by inhibition of the enzyme γ-glutamylcysteine synthetase (51), whereas BCNU interferes with recycling of GSSG into GSH by inhibition of the enzyme GSH reductase (20). Thus, modulation of the kidney GSH redox cycle by two chemically dissimilar compounds worsened renal failure. Taken together, these results provide a strong evidence for the role of GSH in glycerol-induced acute renal failure. It is estimated that about one-third of the patients with rhabdomyolysis will develop acute renal failure (2). The results of our study suggest the possibility of preventing acute renal failure and the associated morbidity and mortality in these patients by using some of the recently described compounds such as GSH esters (13, 28, 40).

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