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Effects of Glutathione on Aminonucleoside Nephrosis in Rats

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Summary. **Several experiments have shown that renal tissue injury by oxidant stress can be lessened by treatment with antioxidant enzymes. Although glutathione (GSH), one of the antioxidants, is widely present in the body, its effects on experimental renal injury have not been fully understood. The effects of glutathione (GSH) on puromycin aminonucleoside (PAN) nephrosis were examined by comparing two groups of rats: those given daily PAN injections (PAN-rats), and those treated with GSH prior to the PAN injection (GSH+PAN-rats). Results showed that: characteristic morphological changes for PAN nephrosis were either not present or very mild in the GSH+PAN-rats; in kidney specimens taken on the 10th day of the experiment, a large number of hyaline droplets and vacuolizations were demonstrated in the PAN-rats but not in the GSH+PAN-rats. With electron microscopy, hyaline droplets and large vacuolizations were marked in glomerular epithelial cells (GECs) from the PAN-rats. In the GSH+PAN-rats, these degenerative changes were not seen, but prominently developing intracellular organellas and Golgi apparatus were demonstrated. Although a marked loss of polyethyleneimine (PEI) particles was demonstrated on glomerular basement membranes (GBM) in both rat groups, the almost complete restoration of PEI particles was demonstrated in the GSH+PAN-rats despite the daily PAN injections for 15 days. Urinary protein excretions increased significantly in both groups; however, they were significantly lower in the GSH+PAN-rats on the 15th day of the experiment. These results suggest that GSH protects the kidney from PAN nephrosis by accelerating the recovery of glomerular epithelial anionic sites.**

Key words—aminonucleoside nephrosis, glutathione.

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INTRODUCTION

Oxidant stress has been suggested to contribute to tissue injury[1]. Several experiments have shown that tissue injury can be lessened by treatment with antioxidant enzymes such as superoxide dismutase and catalases, or by the administration of allopurinol which presumably blocks O2-production by xanthine oxidase. In models of renal ischemia[2,3], the protective effect of allopurinol, as assesed by a marked diminution in the xanthine oxidase-mediated oxidation of hypoxanthine, indirectly demonstrates the contribution of free radicals to renal tissue injury. Additionally, in an acute nephrotoxic nephritis model produced by infusion of an antiglomerular basement membrane antibody, treatment of animals with catalase produced as much as 75% protection against glomerular injury[4]. Thakur et al[5]. have demonstrated a beneficial effect of hydroxyradical scavengers and iron chelators in a model of renal injury.

The aminonucleoside of puromycin (PAN) has been widely used to produce nephrosis in rats[6]. Diamond et al[7]. have suggested a possible role for oxygen free radicals in the development of aminonucleoside nephrosis[7] (PAN nephrosis). The protective effects of superoxide dismutase and allopurinol provides indirect evidence that oxygen free radicals, genenrated by xanthine oxidase, are important mediators of PAN-induced proteinuria[7].

Among various antioxidant substances, tripeptide glutathione (γ-glutamylcysteinyl-glycine, GSH) is the major free thiol in most living cells which participates in such diverse biological processes as the detoxication of xenobiotics, removal of hydroperoxidases, protection against effects of ionizing radiation, and maintenance of the sulfhydryl status of proteins[8].

In the degradation processes of oxidant free radicals to water and oxygen, GSH is oxidized to GSSG

(oxidized form of glutathione) and then reduced back to GSH by glutathione peroxidase. Although several researchers have demonstrated that both GSH and glutathione peroxidase are abundantly present in renal tissue[3,8,9,10,11], the role of glutathione-dependent antioxidant system in PAN nephrosis has not been established. This study was undertaken to clarify whether GSH exerts any effects on the development and course of PAN nephrosis in rats.

MATERIALS AND METHODS

Experimental animals

Male Wistar rats aged 5 weeks weighing 100 g, were used in these experiments. They were allowed free access to food and water.

Drugs and chemicals used

Puromycin aminonucleoside (PAN) and GSH were purchased from Sigma Chem Co. (USA). Other chemicals were of analytical grade and obtained commercially.

Experimental schedules (Fig. 1)

Twenty-three rats were divided into three experimental groups with the following respective three subdivisions.

1)PAN-group: Nine rats received daily subcutaneous injections of PAN at a dose of 1.5 mg/100 g BW, and three each were sacrificed at 5, 10, and 15 days after the initiation of the daily injections. PAN was used as a 0.5% solution dissolved in normal saline.

2)GSH+PAN-group: Nine rats in this group received daily injections of both GSH and PAN according to the schedule (Fig.1). Commercially available GSH (Yamanouchi Pharm Corp Ltd, Japan) was used as a solution of 67 mg/dl for the administration to rats. The intraperitoneal injection of GSH (150 mg/100 g BW) was given 2-3 min prior to the subcutaneous PAN injection.

The cumulative doses of both PAN and GSH given to the rats were 7.5-22.5 mg/100 g of body weight for PAN, and 750-2,250 mg/100 g of BW for GSH, respectively. Although the dosage seemed rather higher than that usually used, the regimens were chosen based on the purpose of this experiment to examine, using near to maximum doses, whether GSH exerts any effects on PAN nephrosis.

3)Control group: Five rats were given normal saline as controls.

Urinary protein concentrations were measured on the final day of each experiment, using freshly voided urine samples. Protein concentrations were determined by a commercially available kit (Protein-Assay, Jap Biorad Lab Co, Japan) and creatinine concentrations by Creatinine-Test Wako (Wako chemicals, Japan)

Hsitological examination

The animals were anesthesized with ether. Observation with PEI was conducted in accordance with the method by Schurer et al.[12] in the following way: Each rat was intravenously injected with 0.1 ml of PEI (MW 1,600) solution/ 100 g BW, 20-30 min prior to sacrifice. After perfusion through the heart with 200 ml of physiological saline, the right kidney was resected for PEI examinations. Renal cortices were cut into small pieces and stained by immersion in 2%

Days	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Control															
PAN-group															
5 days	↓	↓	↓	↓	↓										
10 days	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓					
15 days	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
GSH+PAN-group															
5 days	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓										
10 days	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓					
15 days	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓	⇩↓

↓ Subcutaneous injection of puromycin aminonucleoside (PAN, 1.5 mg/100 g of body weight)

⇩ Intraperitoneal injection of glutathione (GSH, 150 mg/100 g of body weight) 2-3 min prior to the PAN injection.

Fig. 1 Schedule of the experiments.

phosphotungustic acid-0.1% glutaraldehyde mixture for an hour. They were washed three times for 10 min intervals with 0.1M-sodium cacodylate buffer and post-fixed in 1% osmium tetroxide in the cacodyolate buffer at 4 ℃ for 2 h. After dehydration through an ethanol-propylene oxide series, the specimens were embeded in Araldite M. Ultrathin sections were prepared, stained with lead citrate, and examined under a Hitachi M-7000 electron microscope. The left kidney was further perfused with 300 ml of 2.5% glutaraldehyde in 0.1M-sodium cacodylate buffer, pH 7.4 for 5-10 min, and perfused for conventional electron micrograph study. At least twenty sections of each rat were observed by one of our colleagues who had received no information on the treatment for each rat.

Statistical analysis

Urinary protein concentrations were expressed as the mean ±SE. The differences were analyzed based on the Student's unpaired t-test for small samples.

RESULTS

Morphological changes

With light microscopy of semi-thin sections, prominent droplet degenerations and large vacuolar changes were observed in glomeruli from those rats which had been administered with daily PAN injections (PAN-rats); however, these degenerative changes were less severe in the rats treated with GSH prior to the PAN injection (GSH+PAN-rats) (Fig.2). The vacuolar degenerative changes of kidney specimens from the PAN-rats were further advanced on the 15th day of the experiment than on the 10th day of the experiment. In contrast, the observed degenerative changes, though very mild, disappeared in the GSH+PAN rats (Fig.2).

By electron microscopy, hyaline droplet degenerations were observed in glomerular epithelial cells from the PAN-rats taken on the 10th day (Fig.3); these became severer, with an addition of large vacuolizations, in kidney specimens on the 15th day of the experiment (Fig.3). Although a small number of hyaline droplets were present, degenerative vacuolar changes were not observed in the GSH+PAN rats on the 10th or 15th day of the experiment (Fig.4). Furthermore, intracellular organellas and Golgi apparatus were prominently developing in these cells on the 15th day, indicating that active metabolic processes were underway (Fig.4). Almost all glomerular epthelial cells showed swelling with or without a partial fusion of foot processes in the PAN-rats. They remained normal in the GSH+PAN-rats (Fig.5), however.

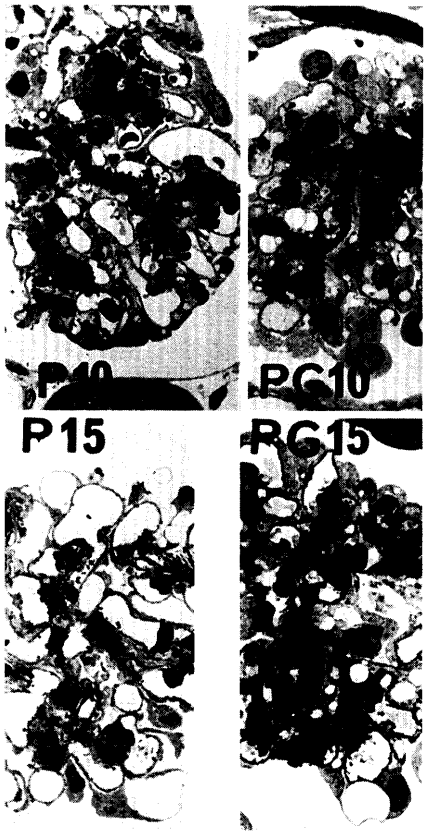

Fig. 2. Light microscopy of semi-thin sections. Hyaline droplets and vacuolizations are present in the glomerulus from PAN-rats taken on the 10th day of the experiment (P10). In the GSH+PAN-rats, only a few hyaline droplets and slight vacuolizations are seen (PG10). Degenerative vacuolizations are more prominently observed in the PAN-rats on the 15th day (P15). In contrast, the observed degenerative changes are recovering and glomerular epithelial cells appear normal in the GSH+PAN-rats (PG15) (Toluidin Blue, × 100).

Changes in anionic sites on glomerular basement membrane (GBM)

Serial changes in PEI particles, representing anionic sites on GBM, are shown in Fig.6. A marked loss of PEI particles on GBM was demonstrated in kidney specimens on the 5th and 10th day of the experiments in both PAN-and GSH+PAN-rats. In the kidney specimens taken on the 10th day, a slight recovery of PEI particles was observed in the GSH+PAN-rats, but not in the PAN-rats. Furthermore, in glomeruli from the rats treated with GSH, an almost complete

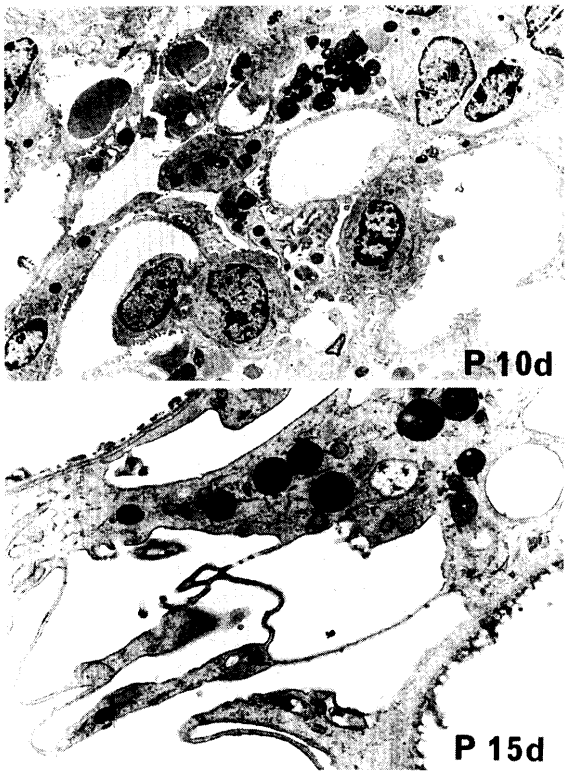

Fig. 3. Electron microscopy.
A large number of hyaline droplets are present in glomerular epithelial cells from PAN-rats taken on the 10th day (P10d). In addition to these findings, large vacuolizations are observed in the PAN-rats taken on the 15th day (P15d).
(uranium acetate for 10 min and lead citrate for 5 min, P10d × 2,800, P15d × 9,800).

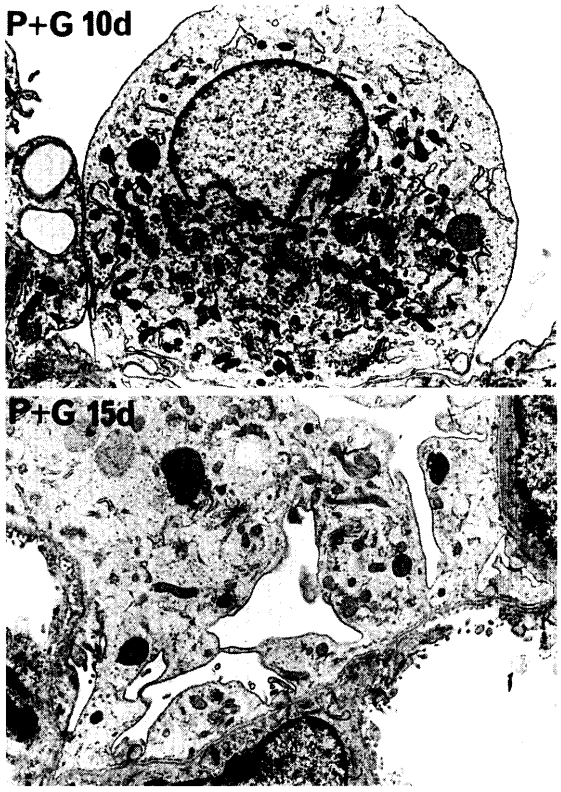

Fig. 4. Electron microscopy.
In the glomerulus from the GSH+PAN-rats taken on the 10th day (P+G10d) and the 15th day (P+G15d), a small number of hyaline droplets are seen. Vacuolar degeneration is not present, however. Intracellular organellas, including Golgi apparatus, are markedly developing in glomerular cells (uranium acetate for 10 min and lead citrate for 5 min, P+G10d × 6,700, P+G15d × 7,300).

restoration of PEI particles was found in the GSH+PAN-rats on the 15th day.

Urinary protein concentrations (Table 1)

Urinary protein concentrations increased significantly both in PAN-and GSH+PAN-rats; however, they were significantly lower in the GSH+PAN-rats on the 15th day of the experiment than in the PAN-rats.

DISCUSSION

This study histologically demonstrated the protective effect of GSH on PAN nephrosis. Urinary protein excretions were lower in GSH-treated rats than in those without GSH treatment.

Although the precise mechanism of PAN nephrosis

Table 1 Urinary protein excretion in rats

	Urinary protein (μg/mg creatinine)
Control (N=5)	172.8±25.1
PAN group (N=9)	
5 days (N=3)	367.6±60.3[a]
10 days (N=3)	1,268.8±58.4[b]
15 days (N=3)	1,738.1±102.1[b]
GSH+PAN-group (N=9)	
5 days (N=3)	344.9±66.4[a]
10 days (N=3)	1,158.9±58.9[b,e]
15 days (N=3)	1,001.0±81.6[b,d]

The values are expressed as mean ±1SE. p values were calculated based on Student's unpaired t-test for small samples: a, $p<0.01$ vs. control; b, $p<0.005$ vs. control; c, $p<0.005$ vs. PAN-group (10 days); and d, $p<0.05$ vs. PAN-group (15 days).

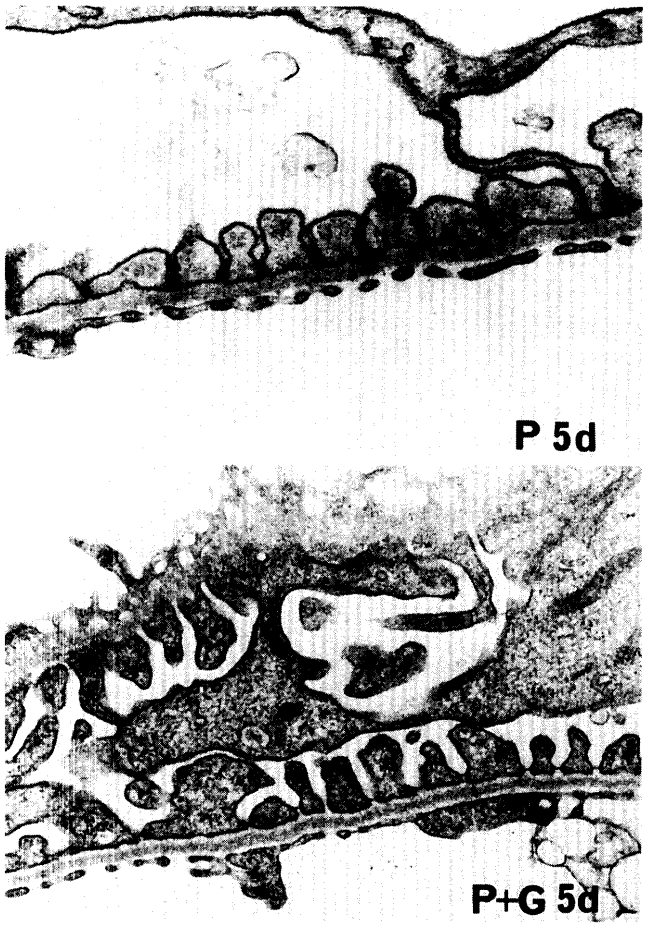

Fig. 5. Electron microscopy.
Swelling and a partial fusion of foot processes are observed in glomerular epithelial cells from the PAN-rats taken on the 5th day (P5d). In contrast, no abnormal changes are seen in the GSH-PAN-rats (P+G5d) (uranium acetate for 10 min and lead citrate for 5 min, × 21,000).

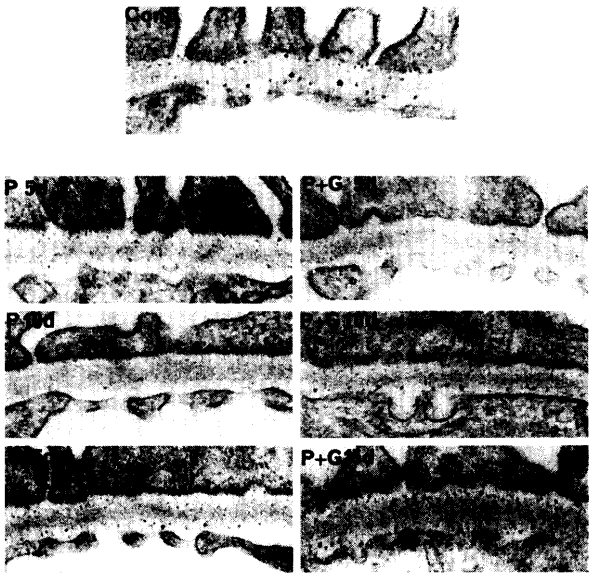

Fig. 6. Changes in glomerular epithelial anionic sites.
A marked loss of PEI particles on the glomerular basement membrane as compared with the control rats (Cont) is observed in kidney specimens taken on the 5th (P5d, P+G5d) and the 10th day (P10d, P+G10d) in both PAN-and GSH+PAN-rats. A slight recovery of PEI particles is seen in the GSH+PAN-rats on the 10th day (P+G10d), but not in the PAN-rats (P10d). An almost complete restoration of PEI particles, near that of the control rats (Cont), is observed in the GSSH+PAN rats on the 15th day (P+G15d). In contrast, only a few PEI particles are seen in the PAN-rats (P15d). (lead citrate for 1 min, × 60,000).

remains unclear, the glomerular epithelial cell has been morphologically demonstrated to be the primary target in renal injury[13]. The *in vivo* sensitivity of glomerular epithelial cells to PAN and the protective effects of PAN antagonists suggest a role for these cells in *in vivo* PAN nephrotoxicity, where alterations in both the morphology and the anionic topology of glomerular epithelial cells participate in the development of proteinuria[14]. A recent report has demonstrated the protective effects of superoxide dismutase on PAN nephrosis, and that oxygen free radicals are important mediators of PAN-induced proteinuria[7]. In the present study, histological changes characteristic of PAN nephrosis[13,15,16,17]were observed in the rats given PAN. However, these were normal or less severe in the animals treated with GSH prior to the PAN injection than in those without GSH treatment. In addition, despite the PAN injections, an almost complete restoration of the lost PEI particles was observed in the animals treated with GSH.

GSH is a tripeptide which plays a significant role in the reduction of disulfides and in the protection of cells from the effects of radiation, oxygen intermediates, and free radicals[8]. Although by the fourth week following PAN administration, spontaneous healing of the morphological appearance of glomeruli was repoted[13], the observed protective effect further suggests that glomerular injury associated with PAN could be lessened by GSH administration.

Since electron microscopy still demonstrated a loss of PEI particles typical for this model[13,14,18]even in the rats treated with GSH, the development of PAN nephrosis, though very mild, could not completely be prevented by GSH.

However, restoration of the PEI particles was very rapid and almost complete in the GSH-treated animals as compared with those given only PAN. These histological improvements also may correspond with the significantly low urinary protein concentrations in the GSH-treated animals.

In conclusion, the results of this study suggest that GSH is effective in protecting kidneys from PAN nephrotoxicity.

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