Stress-Induced Gastric Lesion Formation Is Prevented in Rats With Daunomycin-Induced Nephrosis

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ABSTRACT—In the present study, we investigated the susceptibility to restraint plus water-immersion stress (RWIS) in rats with daunomycin-induced nephrosis in comparison to that in normal rats. The severity of RWIS-induced gastric lesions was significantly less in nephrotic rats on the 20th and 40th days after a single i.v. injection of daunomycin (12 mg/kg) than in the respective control rats. Acid secretion in pylorus-ligated rats significantly decreased under the 3-h stress. On the 20th day after treatment with daunomycin, acid secretion was significantly less in nephrotic rats than in control rats under both stress and unstressed conditions. Pretreatment of normal rats with methylene blue, a guanylate cyclase inhibitor, or phenylephrine, a vasoconstrictor, significantly prevented the stress-induced gastric lesions and decreased acid secretion. *N*^ω-Nitro-L-arginine methyl ester, a nitric oxide (NO) synthase inhibitor, prevented the stress-induced gastric lesion formation only. These results indicate that nephrotic rats are more resistant to RWIS-induced gastric lesions than normal rats. In addition, these results suggest that the decrease in acid secretion related to the decrease in the release of NO from endothelial cells may contribute, at least in part, to the prevention of the stress-induced gastric lesion formation in nephrotic rats.

Keywords: Nephrosis, Stress, Gastroprotection, Gastric secretion

It has been demonstrated that the endothelium-derived NO not only mediates the relaxant responses to a variety of vasodilators (1), but also modulates the vasoconstrictor responses to many agonists (2, 3). Our previous studies have demonstrated that the endothelium-dependent relaxing response to acetylcholine and A23187, a calcium ionophore, is attenuated in aortic rings from rats with daunomycin-induced nephrosis in comparison with the tissues from normal rats (4), while the contractile response to adrenoceptor agonists such as norepinephrine is enhanced (5). Furthermore, more recently, we have shown that nephrotic rats are more susceptible to HCl-ethanol-induced gastric lesions than normal rats and that high susceptibility to the gastric lesions in nephrotic rats may be, at least in part, due to the decrease in gastric mucosal blood flow (6). We have postulated that the decrease in mucosal blood flow observed in nephrotic rats may be attributed to the decrease in the release of NO from endothelial cells because pretreatment of normal rats with *N*^ω-nitro-L-arginine methyl ester, a NO synthase inhibitor, methylene blue,

a guanylate cyclase inhibitor, and phenylephrine, a vasoconstrictor, not only aggravated the HCl-ethanol-induced gastric lesions, but also decreased gastric mucosal blood flow.

Stress ulcers are induced in the stomach in experimental animals and humans after physical or psychological stress (7, 8). Although the pathogenesis of stress-induced gastric lesions is poorly understood, it is considered that the main factors in the lesion formation may be due to increases in acid secretion (9, 10) and gastric motility (11, 12) and decreases in gastric mucosal blood flow (13, 14) and alkaline secretion (15).

In a preliminary study, we found that rats with daunomycin-induced nephrosis were more resistant to RWIS-induced gastric lesion formation than normal control rats. We hypothesized that the decrease in mucosal blood flow mediated by the decrease in the release of NO from capillary endothelial cells may result in the decrease in acid secretion, leading to the prevention of RWIS-induced gastric lesion formation in nephrotic rats. Therefore, in order to test this hypothesis in the present study, we compared the gastric acid secretion of nephrotic rats under both stress and unstressed conditions with that of

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normal rats. Furthermore, we examined the effects of *N*^w-nitro-L-arginine methyl ester, methylene blue and phenylephrine, which decrease gastric mucosal blood flow, on RWIS-induced gastric lesion formation and acid secretion.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley SPF rats (Nihon Clea, Tokyo), weighing 168–175 g, were used in all experiments.

Drugs

Daunomycin (daunorubicin hydrochloride) was purchased from Meiji Seika, Tokyo). *N*^w-nitro-L-arginine methyl ester, methylene blue and L-phenylephrine hydrochloride were purchased from Sigma Chemical (St. Louis, MO, USA). Daunomycin, *N*^w-nitro-L-arginine methyl ester, methylene blue and phenylephrine were dissolved in physiological saline.

Induction of nephrosis

Nephrosis was induced in rats by a single i.v. injection in the tail vein of 12 mg/kg of daunomycin dissolved in physiological saline (16). Age-matched control rats were injected with physiological saline only. To confirm nephrosis, the urinary protein and plasma cholesterol contents were determined by the methods of Kingsbury et al. (17) and Allin et al. (18), respectively.

Induction and measurement of gastric lesions

The RWIS-induced gastric lesions in daunomycin-treated and control rats were evaluated on the 5th, 20th and 40th days after treatment with daunomycin. The animals were deprived of food but not water for 24 h before the initiation of the stress procedures. In accordance with the method of Takagi and Okabe (7), each animal was placed in an individual restraint cage and vertically immersed in a water bath at 23°C to the level of the xyphoid process for 6 h. After the stress load, the animals were killed by overdosage of ether. The stomach of each animal was removed and inflated by injecting 5 ml of 2% formalin to fix the inner and outer layers of the gastric walls. Subsequently, the stomach was incised along the greater curvature. The length and width of each lesion were measured with a micrometer, which was set on a stereoscopic microscope, and the product of both lengths (mm²) was calculated as the area of each gastric lesion. The sum of the area of the lesions per rat was used as the lesion index.

The effects of *N*^w-nitro-L-arginine methyl ester, methylene blue and phenylephrine on RWIS-induced gastric lesions in normal rats were evaluated. These agents were

given i.v. in a volume of 0.5 ml/200 g of body weight 15 min before the stress load. As a control, physiological saline was given i.v. instead of these agents.

Gastric acid secretion

Gastric acid secretion under stress and unstressed conditions in daunomycin-treated and control rats was measured on the 5th and 20th days after treatment with daunomycin. The animals were deprived of food but not water for 24 h before the experiment. The pylorus of each rat was ligated under ether anesthesia. Half of daunomycin-treated and control rats were subjected to RWIS immediately after pylorus ligation. The remaining rats were subjected to pylorus ligation only. The gastric contents were collected at 3 h after ligation. The volume of gastric juice was measured, the acidity was determined by an automatic titrator (ABT-101; Tohadenpa, Tokyo) and total acid output during the 1-h period was calculated.

The effects of *N*^w-nitro-L-arginine methyl ester, methylene blue and phenylephrine on gastric acid secretion were also evaluated by 3-h pylorus ligation. These agents were given intravenously 15 min before pylorus ligation.

Statistical analyses

Results obtained were expressed as the mean ± S.E.M. The data were analyzed by one-way analysis of variance, and the statistical significance among groups was determined by Duncan's multiple-range test or Student's *t*-test. In all cases, *P* < 0.05 was considered significant.

RESULTS

Confirmation of nephrosis

On the 5th day after treatment with daunomycin (12 mg/kg, i.v.), the urinary protein and plasma total cholesterol levels of rats treated with this antibiotic were within normal ranges (Table 1). However, on the 20th and 40th days, all rats treated with daunomycin were nephrotic as defined by severe proteinuria and hypercholesterolemia.

RWIS-induced gastric lesions

The severity of gastric lesions of daunomycin-treated rats after 6 h of RWIS on the 5th day after treatment with the antibiotic was not significantly different as compared to that of age-matched control rats (Fig. 1). However, on the 20th and 40th days, the RWIS-induced gastric lesions were significantly less in daunomycin-treated nephrotic rats than in the respective age-matched control rats (daunomycin-treated: 8.4 ± 1.0 mm² vs control: 21.4 ± 2.8 mm² on the 20th day, *P* < 0.01; daunomycin-treated: 11.8 ± 2.4 mm² vs control: 24.4 ± 4.7 mm², on the 40th day, *P* < 0.05).

As we reported previously, the i.v. administration of

Table 1. Changes in urinary protein excretion and plasma cholesterol levels in control and daunomycin-treated rats

Days	Groups	Urinary protein (mg/24-h urine)	Plasma total cholesterol (mg/dl)
5	Control	6.4±0.6	59.1±6.8
	Daunomycin-treated	4.7±0.7	70.9±3.2
10	Control	7.2±0.5	84.0±5.7
	Daunomycin-treated	49.2±13.9**	80.6±5.3
20	Control	10.0±0.8	77.8±3.0
	Daunomycin-treated	296.4±34.8**	197.6±17.7**
40	Control	10.0±1.3	86.2±10.6
	Daunomycin-treated	315.1±21.5**	227.4±21.6**

Days: Days after treatment with daunomycin. Each data value indicates the mean±S.E.M. of 7 rats. Significantly different from the control, **P<0.01.

N^o-nitro-L-arginine methyl ester (10 mg/kg), methylene blue (80 mg/kg) or phenylephrine (0.1 mg/kg) significantly enhanced HCl-ethanol-induced gastric lesions and reduced gastric mucosal blood flow (6) (data not shown). *N*^o-Nitro-L-arginine methyl ester, methylene blue and phenylephrine significantly prevented the formation of the RWIS-induced gastric lesions when the same doses of these agents were given i.v. to normal rats 15 min before the stress load (Fig. 2).

Gastric acid secretion

On the 5th day after treatment with daunomycin, the RWIS load for 3 h to daunomycin-treated and control rats significantly decreased the volume of gastric juice (stressed control: 1.6±0.2 ml vs unstressed control: 3.4±0.3 ml, P<0.01; stressed daunomycin-treated: 2.1±0.2 ml vs unstressed daunomycin-treated: 3.7±0.5 ml, P<

Fig. 1. Comparison of gastric lesions induced by restraint plus water-immersion stress between control and daunomycin-treated rats. Each column denotes the mean±S.E.M. of 8 rats. Significantly different from the respective control, *P<0.05, **P<0.01.

Fig. 2. Effects of *N*^o-nitro-L-arginine methyl ester (L-NAME), methylene blue and phenylephrine on gastric lesions induced by restraint plus water-immersion stress in rats. Each column denotes the mean±S.E.M. of 8 rats. Significantly different from the respective control, *P<0.05.

Fig. 3. Comparison of gastric acid secretion under restraint plus water-immersion stress and unstressed conditions between control and daunomycin-treated rats. Each column denotes the mean $\pm$ S.E.M. of 7 rats. Significantly different from the respective unstressed rats, $^{\#}P < 0.05$, $^{##}P < 0.01$. Significantly different from the respective control, $^{*}P < 0.05$, $^{**}P < 0.01$.

0.01) and total acid output (stressed control: 52.1 ± 8.8 $\mu\text{eq/h}$ vs unstressed control: 102.9 ± 15.4 $\mu\text{eq/h}$, $P < 0.01$; stressed daunomycin-treated: 50.9 ± 7.1 $\mu\text{eq/h}$ vs unstressed daunomycin-treated: 99.5 ± 24.2 $\mu\text{eq/h}$, $P < 0.05$) measured by 3-h pylorus ligation (Fig. 3). There was no significant difference in the volume of gastric juice and total acid output between daunomycin-treated and control rats under stressed and unstressed conditions. On the 20th day, the volume and total acid output of daunomycin-treated nephrotic and control rats were also decreased by the RWIS load. However, the volume of gastric juice and total acid output were significantly lower in the daunomycin-treated nephrotic rats than in control rats under the unstressed condition (the volume: daunomycin-treated, 4.1 ± 0.3 ml vs control, 4.8 ± 0.3 ml, $P < 0.05$; total acid output: daunomycin-treated, 78.6 ± 11.1 $\mu\text{eq/h}$ vs control, 133.7 ± 13.2 $\mu\text{eq/h}$, $P < 0.01$) (Fig. 3).

Pretreatment of normal rats with methylene blue (80 mg/kg, i.v.) or phenylephrine (0.1 mg/kg, i.v.) sig-

nificantly prevented the volume of gastric juice and total acid output when they were measured by 3-h pylorus ligation (Fig. 4). However, *N*^w-nitro-L-arginine methyl ester (10 mg/kg, i.v.) did not significantly affect both the volume of gastric juice and total acid output.

DISCUSSION

Sternberg (16) reported that a single i.v. injection of daunomycin into rats was able to produce a nephrotic syndrome characterized by massive proteinuria, hyperlipidemia and edema clinically, which was persistent for at least 1 year. Histopathological studies of glomeruli revealed that there was a loss of foot processes in epithelial cells and closely resembled human lipid nephrosis.

In the present study, severe proteinuria and hypercholesterolemia were observed on the 20th and 40th days after treatment with daunomycin, although these changes were not seen on the 5th day.

Fig. 4. Effects of *N*^ω-nitro-L-arginine methyl ester (L-NAME), methylene blue and phenylephrine on gastric acid secretion under unstressed conditions in rats. Each column denotes the mean $\pm$ S.E.M. of 7 rats. Significantly different from the respective control, ** $P < 0.01$.

Our previous studies indicated that rats with daunomycin-induced nephrosis were more susceptible to HCl-ethanol (150 mM HCl in 60% ethanol)-induced gastric lesions than normal rats. Ethanol has been shown to produce gastric lesions by impairment of defensive factors such as mucosal microcirculation (19) and mucus secretion (20). The ethanol-induced gastric lesions are also known to be acid-independent (21). In addition, in the previous experiment, ethanol was orally given to rats together with sufficient acid (150 mM HCl) to eliminate the influence of endogenous acid on gastric lesions. Recently, it was indicated that endothelium-derived NO as well as endogenous prostanoids may play an important role in mucosal microcirculation (22) and mucus synthesis (23). When gastric mucosal blood flow in a previous experiment was measured by the hydrogen gas clearance technique, the blood flow in nephrotic rats on the 40th day after treatment with daunomycin was significantly lower than that in the control animals (control: 40.4 ± 1.2 ml/min per 100 g tissue, vs daunomycin-treated: 28.6 ± 1.2 ml/min per 100 g tissue, $P < 0.05$) (6). However, there was no difference in the blood flow between daunomycin-treated and control rats in the pre-nephrotic stage on the 5th day after treatment. Therefore, we suggested that the aggravation of acidified ethanol-induced gastric lesions observed in nephrotic rats may be, at least in part, due to

the decrease in gastric mucosal blood flow mediated by the decrease in the release of NO from endothelial cells (6). The decrease in the gastric mucosal blood flow in nephrotic rats may cause the aggravation of the gastric lesions by a decrease in gastric mucosal resistance and the disturbance of washing away via the blood flow of a large amount of H^+ diffused into the mucosa from the gastric lumen after the administration of acidified ethanol.

In the present study, we demonstrated that rats with daunomycin-induced nephrosis were more resistant to RWIS-induced gastric lesions than normal rats. Thus, it is interesting that in contrast with the results obtained with acidified ethanol-induced gastric lesions, RWIS-induced gastric lesions were prevented in nephrotic rats. This may be due to the difference in the mechanisms by which acidified ethanol and RWIS induce gastric lesions. As mentioned in the introduction, physical or psychological stress produces ulcers in the stomach of experimental animals and humans (7, 8). The factors in stress-induced gastric lesion formation have been considered to be the increase in acid secretion (9, 10) and gastric motility (11, 12) and the decrease in gastric blood flow (13, 14) and alkaline secretion (15). However, in contrast with increased acid secretion during stress, it has been reported that acid secretion is decreased by stress (24, 25). In the present experiment, we also found that the RWIS load for

3 h caused a significant decrease in the volume of gastric juice and total acid output in nephrotic and control rats, when acid secretion was measured by the 3-h pylorus ligation. Many investigators who reported the decreased acid secretion during stress suggested that acid does not play an important pathogenetic role in stress-induced gastric lesions. Hayase and Takeuchi (25) found in the lumen-perfused rats that the exposure of rats to RWIS decreased acid secretion in the early stage prior to causing gastric lesions up to 2 h after stress load, but there was a marked rise in acid secretion toward normal levels during the aggravating process of the lesions from 3 to 6 h after stress. Therefore, it is postulated in our experiment that acid secretion also enhances in the later stage from 3 to 6 h after the stress load, although we did not measure it in this stage. Furthermore, it is well known that stress-induced gastric lesions are inhibited by antisecretory agents such as antimuscarinic agents, histamine H_2 -receptor antagonists and proton pump inhibitors. These results strongly suggest that acid plays the most important role in the formation of stress gastric lesions.

In the present experiment, in the prenephrotic stage on the 5th day after treatment with daunomycin, the degree of the stress-induced gastric lesions and acid secretion of daunomycin-treated rats was not different from that of control rats. However, in the nephrotic stage on the 20th day after treatment with daunomycin, acid secretion was significantly less in nephrotic rats than in control animals under both stress and unstressed conditions. Therefore, the decreased acid secretion may contribute to low susceptibility to the stress in gastric lesion formation in nephrotic rats. The mechanism by which nephrotic rats decrease acid secretion remains unclear. It has been reported that gastric secretion and mucosal blood flow are directly related (26, 27). Our previous study demonstrated that N^w -nitro-L-arginine methyl ester (10 mg/kg, i.v.), a NO synthase inhibitor, methylene blue (80 mg/kg, i.v.), a guanylate cyclase inhibitor, and phenylephrine (0.1 mg/kg, i.v.), an α_1 -adrenoceptor agonist, aggravated HCl-ethanol-induced gastric lesions in rats and reduced gastric mucosal blood flow (6). In the present study, these three agents prevented the RWIS-induced gastric lesion formation. The results obtained by these agents on acute gastric lesions induced by HCl-ethanol and RWIS were in good agreement with those obtained in nephrotic rats. In addition, at present, methylene blue and phenylephrine decreased acid secretion, although N^w -nitro-L-arginine methyl ester had no apparent effect on it. Noji et al. (28) reported that N^G -nitro-L-arginine (10–50 mg/kg, i.v.), a NO synthase inhibitor, dose-dependently inhibited the acid stimulation by bethanechol, and this inhibition restored by pretreatment with L-arginine. Therefore, low susceptibility to stress in gastric lesions of nephrotic rats

may be, at least in part, due to the reduced acid secretion via the decrease in gastric mucosal blood flow. It has been shown that the reduction of gastric blood flow is an etiology of formation of gastric lesions (10, 16). However, our present study also suggests that the increase in acid secretion, in addition to the reduction of gastric blood flow, may play an important pathogenetic role in RWIS-induced gastric lesions.

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