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## ADAPTIVE CYTOPROTECTION BY AMMONIA AND UREA-UREASE SYSTEM IN THE RAT GASTRIC MUCOSA

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Urease and ammonia ( $\text{NH}_4\text{OH}$ ) have been proposed to play a major role in the pathogenesis of the the *Helicobacter pylori* (*Hp*)-associated gastric damage but the mechanism of this damage has not been fully explained. This study was designed to determine whether topical application with  $\text{NH}_4\text{OH}$  at low concentration or the generation of the  $\text{NH}_4\text{OH}$  in gastric lumen by the hydrolysis of urea in the presence of urease can induce adaptive cytoprotection. Single insult of  $\text{NH}_4\text{OH}$  alone in various concentrations (15—500 mM) caused the mucosal damage starting at 30 mM and reaching at 250 mM the value similar to that obtained with 100% ethanol and being accompanied by the fall in gastric blood flow to about 30% of the normal value. When the mucosa was first exposed to the low concentration (15 mM) of  $\text{NH}_4\text{OH}$ , causing by itself only small microscopic damage of surface epithelium, but then insulted by a high concentration (250 mM) of  $\text{NH}_4\text{OH}$ , the extent of mucosal damage was greatly attenuated as compared to that caused by  $\text{NH}_4\text{OH}$  alone. This “adaptive” cytoprotection, accompanied by the rise in the GBF, was reversed in part, after the pretreatment with indomethacin to inhibit PG-cyclooxygenase, with L-NAME to suppress NO-synthase or with capsaicin to induce deactivation of sensory nerves. The combined topical pretreatment with urea (2%) and urease (100 U) to generate  $\text{NH}_4\text{OH}$  in the stomach, also significantly reduced the severity of gastric lesions induced by 100% ethanol and this was also accompanied by a significant rise in the gastric blood flow. The protective and hyperemic effects of urea and urease were significantly attenuated by the pretreatment with indomethacin or suppression of NO-synthase by L-NAME. The functional ablation of sensory nerves by the pretreatment with capsaicin also reversed, in part, the protective effect of the combination of urea plus urease and abolished completely the mucosal hyperemia accompanying this protection. We conclude that 1)  $\text{NH}_4\text{OH}$  alone at higher concentrations damages the gastric mucosa but when applied at lower concentration corresponding to that in the stomach of *Hp*-infected patients, or generated by the urea in the presence of urease,  $\text{NH}_4\text{OH}$  acts like “mild irritant” to induce adaptive cytoprotection, 2) this adaptive cytoprotection is mediated, in part, by endogenous PG, sensory nerves and arginine-NO-dependent pathway.

**Key words:** ammonia, adaptive cytoprotection, gastric blood flow, gastric adaptation, mucosal proliferation, epidermal growth factor.

## INTRODUCTION

Recent studies postulated that  $\text{NH}_4\text{OH}$ , a product of *Helicobacter pylori* (*Hp*) urease is involved in the pathophysiology of *Hp*-associated gastric mucosal damage (1). *Hp* exhibits a high urease activity and the  $\text{NH}_4\text{OH}$  generated by this enzyme has been shown to have cytotoxic effects on various cultured cell lines in concentration-dependent manner (2,3) and to be noxious for the gastric mucosa in experimental animals (4—6).

It is also possible that the strains of *Hp* in peptic ulcer patients are associated with greater amounts of urease. The remarkably high urease activity of *Hp* seems to play an important role in various gastric disorders associated with *Hp* (5) by enabling the survival of this acid-sensitive microorganism in the gastric mucosa (6). In humans, though the urease activity per organism was similar in *Hp*-infected duodenal ulcer (DU) and in non-DU patients, it was found that both the severity and the activity of gastritis in the antrum were greater in DU compared to non-DU subjects but there was no significant difference in mucosal damage in the body between the two groups (7).

$\text{NH}_4\text{OH}$  generated by hydrolysis of urea was reported to produce severe cytotoxic effects within gastric epithelium *in vitro* (2, 3) and *in vivo* (4, 5, 8—12). The initial response of the gastric mucosa to *Hp* infection was found to be an acute neutrophilic gastritis which progressed to chronic active type B gastritis (13). Such *Hp*-activated neutrophils exhibited damaging effects on the mucosal cells that were attenuated by antineutrophil serum, urease inhibitors or taurine, a scavenger of hypochlorous acid (13—16).

Although the deleterious effects of  $\text{NH}_4\text{OH}$  on gastric mucosa have been confirmed by showing the mucosal damage by the  $\text{NH}_4\text{OH}$  derived from the urea in the presence of the urease in the animal stomach (17), it is not clear, whether the  $\text{NH}_4\text{OH}$ , naturally generated in the stomach of *Hp*-positive patients, is truly cytotoxic. Some evidences suggest that the products of *HP*-urease may, in fact, be beneficial for the host gastric mucosa (12) as well as for the microorganism itself.

The purpose of this study was; 1) to determine the macroscopic and microscopic changes induced by the topical  $\text{NH}_4\text{OH}$  alone in the rat stomach; 2) to assess the possible "mild irritant" or adaptive cytoprotective abilities of topical  $\text{NH}_4\text{OH}$ , 3) to evaluate the effectiveness of urea-urease system in protection against mucosal damage induced by absolute ethanol and 4) to determine the implication of endogenous PG, NO-arginine pathway, histamine  $\text{H}_2$ -receptors, and afferent nerves in  $\text{NH}_4\text{OH}$  and urea-urease-induced cytoprotection.

## MATERIAL AND METHODS

*Production of acute gastric damage by topical NH<sub>4</sub>OH*

Three major series (A, B and C) of Wistar rats either sex, weighing 180—220 g were used in all studies. Series A was employed to determine the damaging effects of NH<sub>4</sub>OH given in graded concentration on the gastric mucosa and changes in the gastric blood flow. In rats of series B — the possible adaptive cytoprotective effects of topical NH<sub>4</sub>OH applied at low concentration against the damage induced by strong irritant concentration of NH<sub>4</sub>OH were assessed. Rats of series C were pretreated with either urea, urease alone or with the combination of urea and urease to generate NH<sub>4</sub>OH intragastrically in order to assess the role of urea-urease system in the protection against NH<sub>4</sub>OH-induced gastric mucosal damage.

Acute gastric lesions in rats of A series were induced by various concentrations of NH<sub>4</sub>OH ranging from 15 mM up to 500 mM applied i.g. NH<sub>4</sub>OH was dissolved in water and given i.g. in a volume of 1.5 ml using an orogastric tube. For comparison, 100% ethanol was also administered i.g. in a volume of 1.5 ml in separate group of rats. In control animals, the animals were given water with pH adjusted (by adding 100 mM NaOH) to the same values as those recorded in solutions of NH<sub>4</sub>OH applied i.g. in various concentrations. The GBF was determined by hydrogen gas clearance in all these experiments as described previously (18). One hour after the application of NH<sub>4</sub>OH, the rats were lightly anesthetized with ether and the GBF was measured in the oxyntic portion of the stomach. Then, the stomach was removed and the area of gastric lesions was measured by planimetry (Morphomat, Carl Zeiss, Berlin, Germany) (18,19). The venous blood samples were taken for the determination of plasma gastrin level as described elsewhere (20). The standardized specimens were taken horizontally through the entire gastric wall (0.5 cm away from the forestomach) for histological examination. The gastric tissue was placed on filter paper with the mucosa upwards and fixed with buffered formalin. Paraffin sections were routinely stained with hematoxylin and eosin. A Nikon microscope equipped with a microplan II was used for the quantitative histological examination (morphometry) of the sections. The deeper necrotic lesions penetrating the mucosa were measured and expressed as percent of total mucosal length of damage. All quantitative histological examinations were performed by the person unaware of the treatment given.

In some test the mucosal samples of the oxyntic gland area were taken by biopsy (about 50 mg) immediately after the animals were killed, to determine the mucosal generation of PGE<sub>2</sub>, by specific radioimmunoassay (RIA) as described previously (19,20). The mucosal sample was placed in preweighed Eppendorf vial, and 1 ml of Tris buffer (50 mM, pH 3.5) was added to each vial. The samples were finally minced (about 15 sec) with scissors, washed, and centrifuged for 10 sec, the pellet being resuspended again in 1 ml of Tris buffer. Then each sample was incubated on a Vortex mixer for 1 min and centrifuged for 15 sec. The pellet was weighed, and the supernatant was transferred to a second Eppendorf vial containing indomethacin (10 nM) and kept at -50 C° until RIA. PGE<sub>2</sub> was measured in duplicate using RIA kits (New England Nuclear, Munich, Germany). The capability of the mucosa to generate PGE<sub>2</sub> was expressed in nanogram of wet tissue weight.

*Studies on adaptive cytoprotective activity of NH<sub>4</sub>OH. The influence of endogenous PG, NO, afferent nerves, histamine, sulfhydryls and polyamines*

Rats of series B were used for the assessment of the possible mucosal adaptive cytoprotection by NH<sub>4</sub>OH given i.g. in low concentration, which by itself caused only negligible mucosal damage observed only microscopically. In one (control) group, gastric lesions were induced by i.g. application of 1.5 ml of NH<sub>4</sub>OH solution in a standard (damaging) concentration of 250 mM. In

another groups, low concentration of  $\text{NH}_4\text{OH}$  (15 mM) was applied either alone or 30 min prior to the administration of  $\text{NH}_4\text{OH}$  at high concentration (250 mM). To test the possible involvement of endogenous PG in adaptive cytoprotection induced by low (15 mM) against high (250 mM) concentration of  $\text{NH}_4\text{OH}$ , rats were pretreated (90 min before the application of  $\text{NH}_4\text{OH}$ ) with indomethacin applied i.p. in a dose of 5 mg/kg that was shown before to suppress the generation of endogenous PG in the mucosa using specific radioimmunoassay as described before (19, 20). One hour, later when 15 mM  $\text{NH}_4\text{OH}$  was applied i.g., the animals were anesthetized with ether to determine the GBF using the  $\text{H}_2$ -gas clearance technique as in previous series of rats.

Several groups of 8—10 rats were employed and the following treatments were given; 1) vehicle (saline), 2) 15 mM or 250 mM  $\text{NH}_4\text{OH}$  alone, 3) 15 mM  $\text{NH}_4\text{OH}$  followed 30 min later by 250 mM  $\text{NH}_4\text{OH}$ , 3) indomethacin (5 mg/kg i.p.) alone, 4) indomethacin (5 mg/kg i.p.) followed 90 min later by 250 mM  $\text{NH}_4\text{OH}$ , 5) indomethacin (5 mg/kg i.p.) followed 90 min later by 15 mM  $\text{NH}_4\text{OH}$  and then after another 30 min by 250 mM  $\text{NH}_4\text{OH}$ .

Deactivation of primary afferent nerves was achieved using capsaicin (Fluka, Buchs, Switzerland) injected s.c. (under ether anesthesia) for 3 consecutive days at a dose of 25, 50 and 50 mg/kg (total of 125 mg/kg) about 2 weeks before the experiment as described previously (21,22). To check for the effectiveness of capsaicin denervation, a drop of a 0.1 mg/ml solution of capsaicin was instilled into one eye of each rat and the protective wiping movements were counted (22). All capsaicin-pretreated rats showed negative wiping movement test. Rats with functional ablation of capsaicin-sensitive nerves and control rats with intact sensory nerves were pretreated with low (15 mM) concentration of  $\text{NH}_4\text{OH}$  followed 30 min later by high concentration (250 mM) of this irritant.

The implication of endogenous nitric oxide (NO) in gastric adaptive protection induced by  $\text{NH}_4\text{OH}$  was studied in rats pretreated with a selective NO synthase inhibitor, L- $\text{N}^{\text{G}}$ -nitro-L-arginine-methyl ester (L-NAME) administered i.v. in a dose of 100  $\mu\text{mol/kg}$ . L-NAME was given either alone or in the combination with L-arginine (2 mmol/kg i.v.), a substrate for the constitutive NO-synthase or D-arginine, which is not a NO-supplier. Injection with L-NAME without or with L-arginine or D-arginine was performed 15 min before the application of a standard concentration (250 mM) of  $\text{NH}_4\text{OH}$  or before the combination of lower concentration of  $\text{NH}_4\text{OH}$  (15 mM) followed 30 min later by higher concentration of  $\text{NH}_4\text{OH}$  (250 mM). Control rats received respective vehicles.

To examine whether  $\text{H}_2$ -receptors are involved in  $\text{NH}_4\text{OH}$ -induced adaptive cytoprotection, ranitidine, a specific  $\text{H}_2$ -receptor antagonist was employed in a dose of 40 mg/kg s.c. that was shown before to produce complete achlorhydria in the stomach (20, 23, 24).

Several groups of rats were used to receive the following treatments; 1) vehicle (saline) followed 30 min later by  $\text{NH}_4\text{OH}$  (250 mM), 2) 15 mM  $\text{NH}_4\text{OH}$  followed 30 min later by 250 mM  $\text{NH}_4\text{OH}$ , 3) ranitidine (40 mg/kg s.c.) followed 60 min later by 250 mM  $\text{NH}_4\text{OH}$ , 4) ranitidine 40 mg/kg s.c. followed 60 min later by 15 mM and then 30 min later by 250 mM  $\text{NH}_4\text{OH}$ , 5) NEM (20 mg/kg s.c.) followed 30 min later by 250 mM  $\text{NH}_4\text{OH}$ , 6) NEM (20 mg/kg s.c.) followed 30 min later by 15 mM  $\text{NH}_4\text{OH}$  and then another 30 min later by 250 mM  $\text{NH}_4\text{OH}$ , 7) DFMO (400 mg/kg i.p.) followed 120 min later by 15 mM  $\text{NH}_4\text{OH}$  and then 30 min later by 250 mM  $\text{NH}_4\text{OH}$ .

### *Evaluation of the role of urea-urease system in gastroprotection against ethanol-induced damage*

Rats of series C were subjected to study whether  $\text{NH}_4\text{OH}$  produced in the stomach by the hydrolysis of urea in the presence of urease acts as a mild irritant and prevents the gastric mucosa from the damage induced by 100% ethanol. Rats were pretreated with either urea (0.5%—6%),

urease (100 U) given alone or with the combination of urea and urease followed 30 min later by 100% ethanol applied i.g. in a volume of 1.5 ml. The involvement of endogenous PG or NO biosynthesis in the protective action of urea-urease system against ethanol-damage was examined by using the pretreatment with indomethacin or L-NAME without or with L- or D-arginine according to the same experimental schedule as described for rats of series B. Separate groups of rats with intact or functionally ablated sensory nerves by capsaicin were subjected to determine whether primary afferent sensory nerves are involved in gastroprotection induced by the urea and urease applied together. In some tests, the effect of concurrent administration of acetohydroxamic acid, a potent urease inhibitor (12), given with the combination with urea plus urease and followed 30 min later by 100% ethanol was examined. One hour afterwards, the gastric lesions and changes in the gastric blood flow were recorded in a similar manner as described in rats of series A and B.

## RESULTS

### *Effect of NH<sub>4</sub>OH on gastric mucosal integrity and GBF*

As shown in *Table 1*, topical NH<sub>4</sub>OH applied in various concentrations ranging from 15 mM up to 500 mM resulted in concentration-dependent increase in the formation of gastric lesions reaching at 250 mM the area similar to that produced by i.g. administration of 100% ethanol. The damaging effect of gradually increasing concentrations of NH<sub>4</sub>OH was accompanied by a progressive fall in the GBF. NH<sub>4</sub>OH given at the concentration of 15 mM, produced the significant decrease in the GBF but failed to show visible mucosal damage in the gastric mucosa (*Table 1*). Histologically, at lower

*Table 1.* Area of gastric lesions and gastric blood flow in rats given i.g. various concentrations of NH<sub>4</sub>OH or 100% ethanol. Means ( $\pm$ SEM) of 8–10 rats. Asterisk indicates significant change as compared with the vehicle (saline) control.

	Mean lesion area (mm <sup>2</sup> )	GBF (ml/min-100 g)
VEH	—	54 $\pm$ 4
NH <sub>4</sub> OH (mM i.g.) 15	3 $\pm$ 0.5	38 $\pm$ 3 *
30	8 $\pm$ 2 *	35 $\pm$ 4 *
60	15 $\pm$ 3 *	22 $\pm$ 3 *
125	53 $\pm$ 6 *	20 $\pm$ 2 *
250	77 $\pm$ 8 *	18 $\pm$ 1 *
500	105 $\pm$ 12 *	15 $\pm$ 0.5 *
100% ETHANOL (i.g.)	89 $\pm$ 9 *	21 $\pm$ 3 *

concentrations of NH<sub>4</sub>OH (15 and 30 mM) only occasional focal desquamation of surface epithelium and dilation of submucosal, intramuscular and subserosal venules were observed (*Table 2*). With increasing concentrations (60 mM or

higher), the submucosal and muscular venules remained dilated and marked submucosal oedema and infiltration by numerous neutrophils were observed. Surface epithelium was massively exfoliated and small superficial hemorrhagic erosions were seen. With further increase of  $\text{NH}_4\text{OH}$  concentrations, the mucosa showed oedema and numerous hemorrhagic necrosis involving up to 80% of the mucosal strip and penetrating the entire mucosa.

Table 2. Summary of gastric wall histology after i.g. administration of  $\text{NH}_4\text{OH}$

$\text{NH}_4\text{OH}$	15	30	60	125	250	500
Surface epithelium	occasional focal desquamation		2—5 foci of desq.	1—20% necr.	20—50% necr.	30—90% necr.
Mucosa	N	occasional focal eosin. necr. of superficial lamina propria or tiny hemor. erosions	focal necrosis of superf. lamina propria or superf. erosions	marked hemor. necrosis	idem	idem
Mucosal collecting venules	N	vasodil.	marked vasodil.	idem	idem	idem
Muscularis mucosa veins	N	vasodil.	marked vasodil.	idem	idem	idem
Submucosa	N	marked edema, vasodil. leuk. infiltr.	marked edema vasodil. and leukocytes	idem	idem	idem
Muscularis propria and subserosal veins		vasodil.	idem	idem	idem	idem

N = normal; desq. = desquamation; necr. = necrosis; superf. = superficial; infiltr. = infiltration; accumul. = accumulation; hemor. = hemorrhage; vasodil. = vasodilation; eosin. = eosinophilic; leuk. = leukocytes or leukocytic.

*Demonstration of adaptive gastroprotection by topical NH<sub>4</sub>OH*

Table 3 shows the effect of topical pretreatment with NH<sub>4</sub>OH at low concentration (15 mM) on the area of gastric lesions and the changes in the GBF induced by NH<sub>4</sub>OH at high concentration (250 mM). Low concentration of NH<sub>4</sub>OH (15 mM i.g.) by itself failed to induce gross gastric lesions though, as mentioned previously, histologically a focal exfoliation of surface epithelium was observed (data not shown). GBF was significantly reduced by about 15% as compared to the initial vehicle-pretreatment value. NH<sub>4</sub>OH applied on the gastric mucosa at the concentration of 250 mM produced a widespread hemorrhagic mucosal lesions and this was accompanied by a significant decline in the GBF but without alteration in mucosal generation of PGE<sub>2</sub>. Pretreatment with NH<sub>4</sub>OH applied i.g. in low concentration (15 mM) 30 min before 250 mM NH<sub>4</sub>OH, resulted in the area of gastric lesions that was reduced by about 50% as compared to that observed after 250 mM NH<sub>4</sub>OH alone and this was accompanied by a significant rise in the GBF but without the significant change in the mucosal generation of PGE (Table 3).

Table 3. Area of gastric lesions, gastric blood flow and mucosal generation of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in rats treated with ammonia (NH<sub>4</sub>OH) at lower concentration (15 mM), higher concentration (250 mM) or their combinations in tests without or with pretreatment with indomethacin (5 mg/kg i. p.) or L-NAME (100 µmol/kg i. v.) without or with addition of L-arginine or D-arginine (2 mmol/kg i. v.). Means (±SEM) of 8–10 rats.

TREATMENT	LESION AREA (mm <sup>2</sup> )	GBF ml/min-100 g	PGE <sub>2</sub> (ng/g)
INTACT (CONTROL)	0	57.3 ± 4.1	370 ± 59
15 mM NH <sub>4</sub> OH	4.7 ± 1.5	43.3 ± 3.6 *	398 ± 42
250 mM NH <sub>4</sub> OH	74.3 ± 12.7 *	26.2 ± 4.5 *	376 ± 56
15+250 mM NH <sub>4</sub> OH	30.1 ± 5.9 * <sup>+</sup>	38.7 ± 4.1 <sup>+</sup>	382 ± 48
INDOMETHACIN (5 mg/kg i. p.)	4.7 ± 1.2	37.4 ± 4.2 *	74 ± 16 <sup>#</sup>
INDO+15 mM NH <sub>4</sub> OH	6.7 ± 1.5	37.2 ± 4.2	65 ± 12 <sup>#</sup>
INDO+250 mM NH <sub>4</sub> OH	75.1 ± 8.7 *	20.2 ± 2.6 *	72 ± 9 <sup>#</sup>
INDO+15+250 mM NH <sub>4</sub> OH	49.6 ± 4.2 * <sup>+</sup>	37.8 ± 5.6 * <sup>+</sup>	64 ± 8 <sup>#</sup>
L-NAME (100 µmol/kg i. v.)	3.1 ± 1.9	42.1 ± 5.1	NT
L-NAME+15+250 mM NH <sub>4</sub> OH	64.6 ± 9.2	17.8 ± 3.4	NT
L-ARG+15+250 mM NH <sub>4</sub> OH	37.2 ± 5.1	77.2 ± 10.4	NT
D-ARG+15+250 mM NH <sub>4</sub> OH	77.9 ± 11.4 <sup>#</sup>	18.3 ± 3.6 <sup>#</sup>	NT

\* indicates significant change as compared to the value obtained with lower concentration of NH<sub>4</sub>OH (15 mM).

<sup>#</sup> indicates significant change as compared to the value obtained in similar tests on rats without pretreatment with indomethacin or L-NAME.

<sup>+</sup> indicates significant change as compared to the value obtained with larger concentration of NH<sub>4</sub>OH (250 mM).

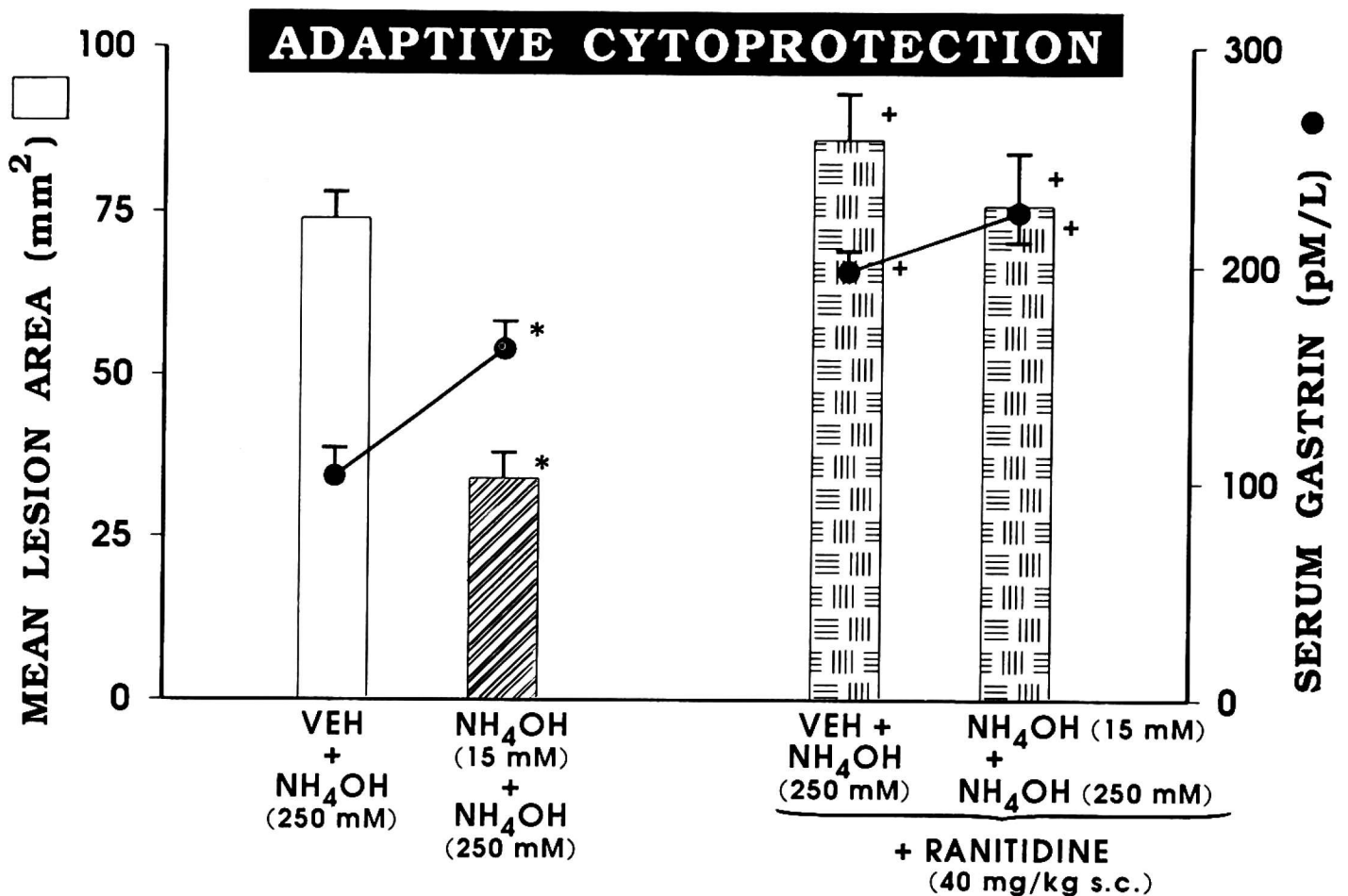
In rats pretreated with indomethacin, the mucosal generation of PGE was suppressed by about 85% as compared to that in vehicle-treated gastric mucosa (data not shown).  $\text{NH}_4\text{OH}$  at low concentration (15 mM) and high concentration (250 mM) administered into indomethacin-treated rats resulted in gastric mucosal lesions and GBF similar to those observed in rats without such treatment. Pretreatment with indomethacin reversed, in part, the reduction in the area of gastric lesions and the increase in the GBF produced by the combination of low (15 mM) plus high concentration (250 mM) of  $\text{NH}_4\text{OH}$  (Table 3).

The pretreatment with L-NAME (100  $\mu\text{mol/kg}$  i.v.), to suppress the biosynthesis of NO (data not shown), resulted in a significant increase in the area of mucosal lesions induced by larger concentration of  $\text{NH}_4\text{OH}$  (250 mM) and almost completely reversed the protective effect of 15 mM  $\text{NH}_4\text{OH}$  against the damage caused by 250 mM  $\text{NH}_4\text{OH}$  (Table 3). The reversal of L-NAME of the protective effects of  $\text{NH}_4\text{OH}$  applied at lower concentration against the damaging action of 250 mM  $\text{NH}_4\text{OH}$  was associated with the similar reduction in gastric blood flow to that observed with 250 mM  $\text{NH}_4\text{OH}$  alone. Concomitant treatment with L-arginine, but not with D-arginine, restored completely the protective and hyperemic effects induced by  $\text{NH}_4\text{OH}$  at low concentration (15 mM) against the damage evoked by  $\text{NH}_4\text{OH}$  at higher concentration (250 mM) (Table 3).

Gastric acid secretion following the pretreatment with  $\text{H}_2$ -receptor antagonist, ranitidine given in a dose (40 mg/kg s.c. that caused almost complete achlorhydria (data not shown) resulted in the augmentation of the area of gastric damage induced by 250 mM  $\text{NH}_4\text{OH}$  as compared to that attained that concentration of  $\text{NH}_4\text{OH}$  in vehicle-pretreated animals (Fig. 1). The reduction in the area of gastric lesions achieved by low concentration of  $\text{NH}_4\text{OH}$  (15 mM) applied i.g. 30 min before 250 mM  $\text{NH}_4\text{OH}$ , was completely abolished by the pretreatment with ranitidine.

The effect of topical application of 250 mM  $\text{NH}_4\text{OH}$  alone or the combination of 15 mM  $\text{NH}_4\text{OH}$  plus 250 mM  $\text{NH}_4\text{OH}$  on the serum gastrin level in rats without or with pretreatment with ranitidine is shown in Fig. 1. The serum gastrin level, which in vehicle-control rats (without administration of  $\text{NH}_4\text{OH}$ ) averaged  $43 \pm 5$  pM/L, was significantly raised by about 2 folds after topical administration of  $\text{NH}_4\text{OH}$  at high concentration (250 mM). This increment in serum gastrin level was further significantly augmented in rats given the combination of 15 mM  $\text{NH}_4\text{OH}$  plus 250 mM  $\text{NH}_4\text{OH}$ . Following the inhibition of gastric secretion by ranitidine (40 mg/kg s.c.) at the dose causing almost complete achlorhydria, a further significant increase in the serum gastrin level in rats treated with 250 mM  $\text{NH}_4\text{OH}$  alone or the combination of 15 mM  $\text{NH}_4\text{OH}$  plus 250 mM  $\text{NH}_4\text{OH}$  was observed as compared to respective controls without ranitidine pretreatment.





*Fig. 1.* Area of gastric lesions and serum gastrin levels in rats pretreated with vehicle or ammonia (NH<sub>4</sub>OH) at low concentration (15 mM) followed by ammonia at high concentration (250 mM) without or with pretreatment with ranitidine (40 mg/kg s.c.). Means ( $\pm$ SEM) of 8–10 rats. Asterisk indicates significant decrease as compared to the vehicle control, while cross indicates significant increase as compared to the vehicle control.

The effects of functional ablation of sensory nerves by capsaicin on gastric lesions induced by 250 mM NH<sub>4</sub>OH applied i.g. either alone or in the combination with 15 mM NH<sub>4</sub>OH is shown in *Fig. 2*. Such pretreatment with capsaicin by itself increased the area of NH<sub>4</sub>OH induced gastric lesions and produced a significant decrease in the GBF as compared to those in rats without capsaicin induced sensory denervation. The protective and hyperemic effects of low concentration of NH<sub>4</sub>OH (15 mM) against the mucosal damage and the accompanying fall in the GBF provoked by high concentration (250 mM) of this irritant were significantly attenuated in rats with capsaicin-induced deactivation of sensory nerves.

#### *Effect of urea-urease system on ethanol-induced mucosal damage and gastric blood flow*

Topical administration of urea and urease either alone or their combination did not produce any visible mucosal lesions in the gastric mucosa and these results have not been presented for the sake of clarity. In contrast, 100%

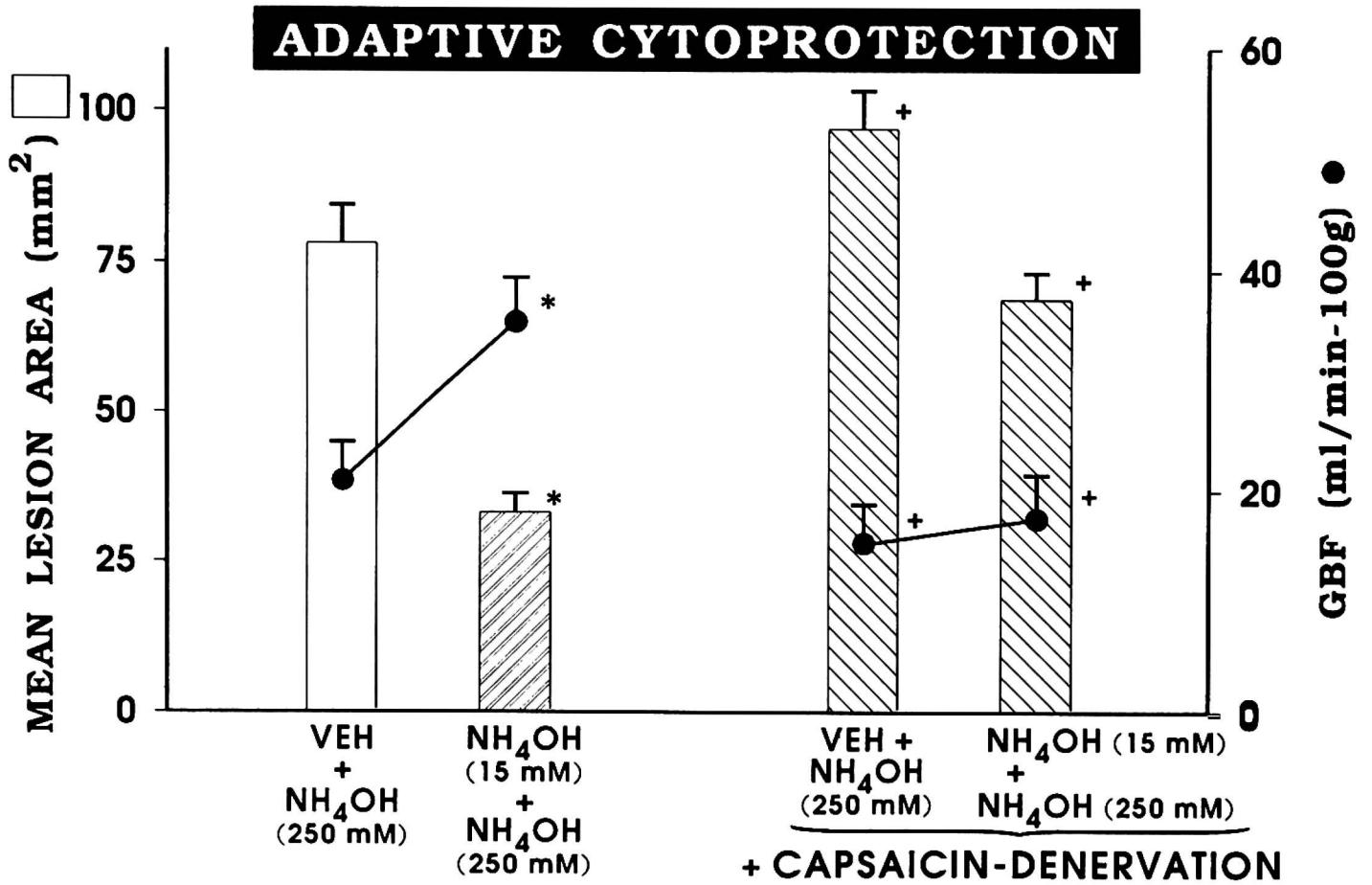


Fig. 2. Area of gastric lesions and gastric blood flow induced by larger concentration of ammonia (250 mM) in rats pretreated with vehicle or lower concentration of ammonia (15 mM) in rats with intact and those with capsaicin-deactivated sensory nerves. Means  $\pm$  SEM of 8–10 rats. Asterisk indicates a significant change as compared to the values recorded in vehicle controls. Cross indicates significant change as compared to the values obtained in respective tests in animals with intact sensory nerves.

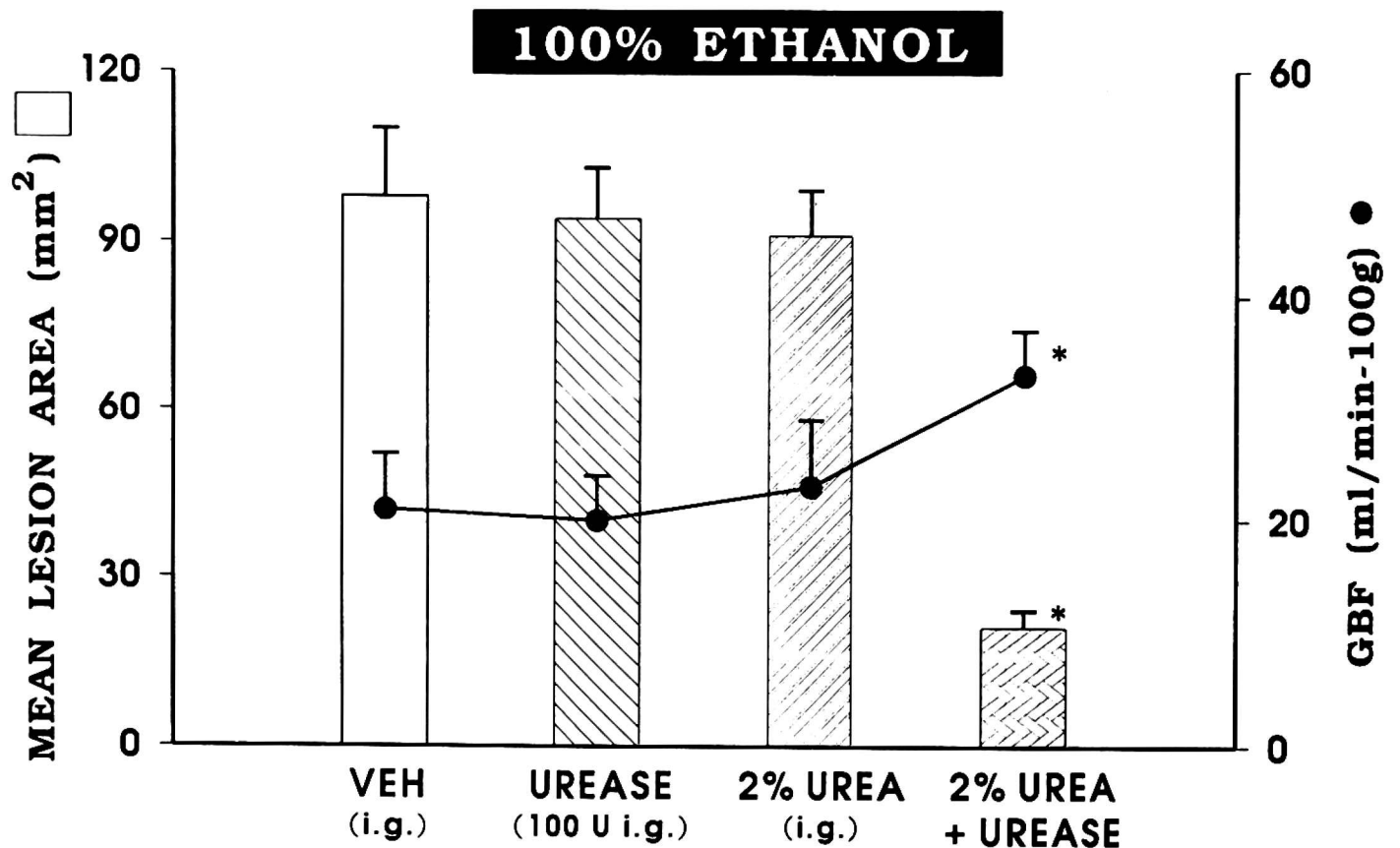


Fig. 3. Area of gastric lesions and gastric blood flow induced by 100% ethanol in rats pretreated with vehicle, urea (2%), urease (100 U) or the combination of urea (2%) plus urease (100 U). Means  $\pm$  SEM of 8–10 rats. Asterisk indicates significant change as compared to values recorded in vehicle-pretreated rats.

ethanol applied i.g. produced widespread mucosal lesions and the mean area of these lesions averaged  $92 \pm 8$  mm (Fig. 3). The GBF in these rats was reduced by about 60% as compared to that in the intact gastric mucosa. Topical pretreatment with urea (2%) or urease (100 U) alone failed to affect the lesions provoked by 100% ethanol and accompanying fall in the GBF (Fig. 3). When the animals were pretreated with the combination of urea (2%) and urease (100 U) and then 100% ethanol was applied i.g., the area of ethanol-lesions was significantly reduced by about 76% and this was accompanied by a significant rise in the GBF as compared to respective vehicle-control rats.

The pretreatment with indomethacin to suppress PG-cyclooxygenase or L-NAME to inhibit NO-synthase produced slight but significant augmentation in ethanol damage and significantly reduced the GBF as compared to those measured in rats receiving 100% ethanol alone (Fig. 4 and 5). Such pretreatment with indomethacin reduced significantly the protection and

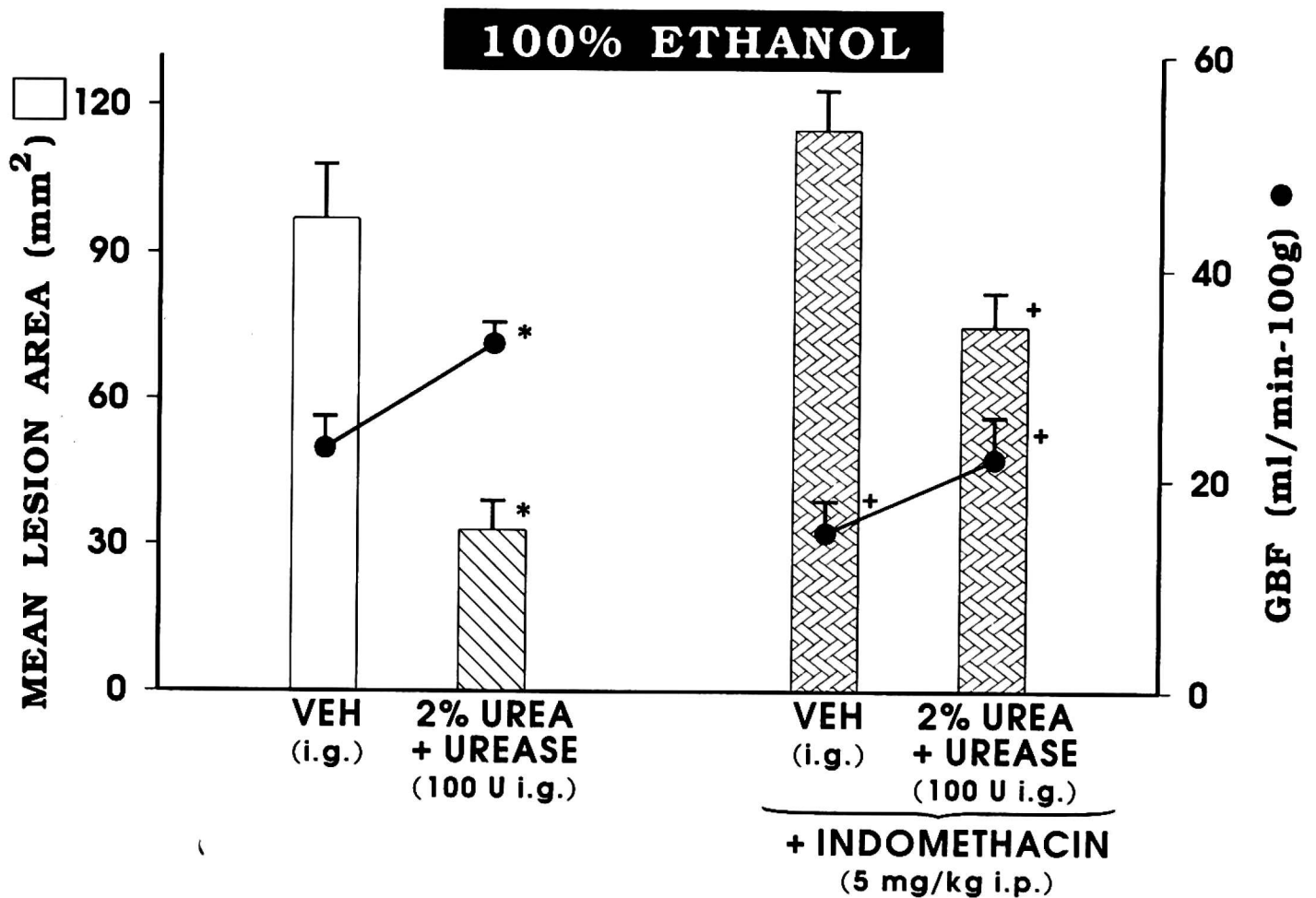
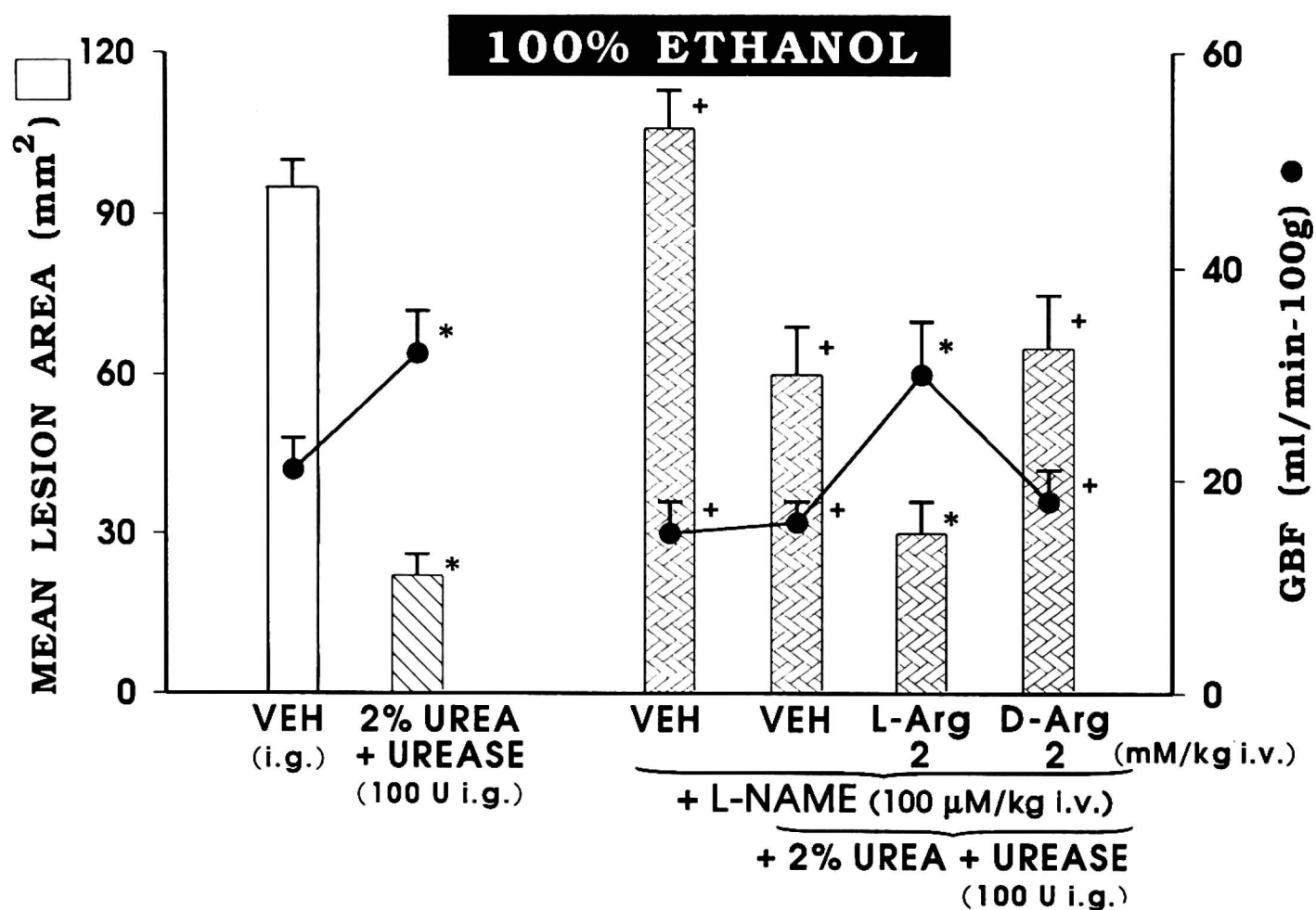


Fig. 4. Area of gastric lesions and changes in gastric blood flow induced by 100% ethanol in rats given i.g. vehicle or the combination of urea (2%) or urease (100 U) without or with pretreatment with indomethacin (5 mg/kg i.p.). Means  $\pm$  SEM of 8–10 rats. Asterisk indicates significant changes as compared to values obtained in vehicle-controls. Cross indicates significant change as compared to that obtained in respective tests without pretreatment with indomethacin.

accompanying rise in GBF caused by concomitant administration of urea and urease (Fig. 4). When L-NAME (100  $\mu$ mol/kg i.v.) was injected before the combination of urea and urease, the area of ethanol-gastric injury was

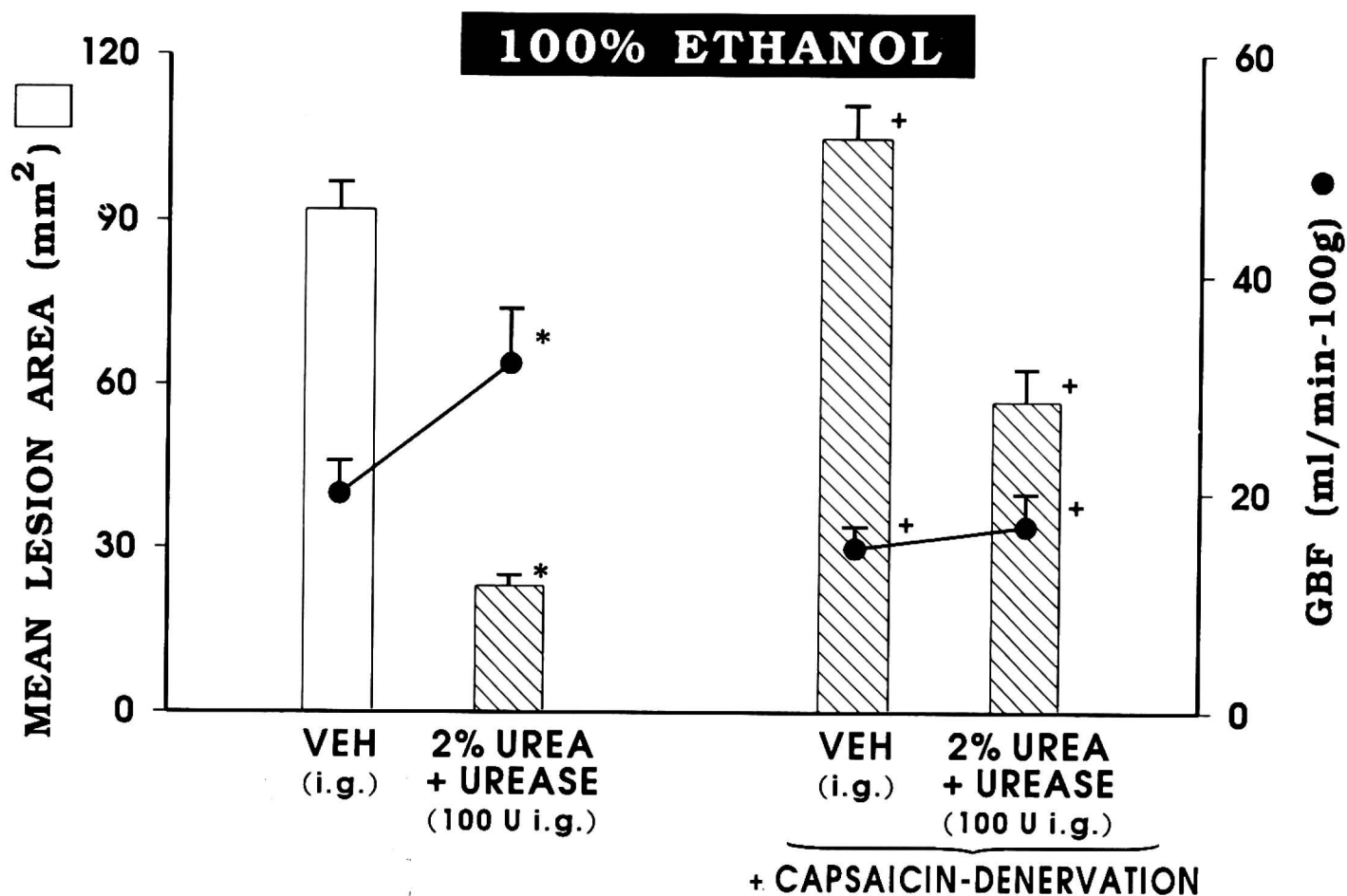
significantly increased and the GBF was significantly attenuated as compared with those obtained in rats treated with urea plus urease without L-NAME pretreatment (*Fig. 5*). The concomitant treatment with L-arginine, but not that with D-arginine, restored completely the protective and hyperemic effects of urea and urease in rats with ethanol-induced gastric lesions.



*Fig. 5.* Area of gastric lesions and changes in gastric blood flow induced by 100% ethanol in rats pretreated with vehicle or the combination of urea (2%) plus urease (100 U) without or with pretreatment with NO synthase inhibitor (L-NAME) (100  $\mu$ mol/kg i.v.) and addition of L-arginine or D-arginine (2 mmol/kg i.v.). Means ( $\pm$ SEM) of 8–10 rats. Asterisk indicates significant change as compared to vehicle control. Cross indicates significant change as compared to the values obtained in respective tests without pretreatment with L-NAME.

*Fig. 6.* shows the effect of the pretreatment of the gastric mucosa with the combination of urea plus urease on the formation of gastric lesions induced by 100% ethanol in rats with intact and those with capsaicin deactivated sensory nerves. In this series of experiments on rats with intact sensory nerves, the combined treatment with urea and urease resulted in a similar protection against damage induced by ethanol and accompanying hyperemia as that shown on *Fig. 3*. In contrast, rats with capsaicin-induced deactivation of sensory nerves exhibited a larger area of gastric lesions followed by a significant decrease in the GBF comparing to the respective values recorded

in animals with intact sensory nerves. The protective and hyperemic effects of the combination of urea plus urease against ethanol damage were significantly reduced in rats with capsaicin-induced deactivation of sensory nerves (*Fig. 6*).



*Fig. 6.* Area of gastric lesions and changes in gastric blood flow induced by 100% ethanol in rats pretreated with vehicle or the combination of urea (2%) plus urease (100 U) in rats with intact sensory nerves or those with capsaicin-induced denervation. Mean  $\pm$  SEM of 8–10 rats. Asterisk indicates significant change compared to vehicle control. Cross indicates significant change compared to the values recorded in rats with intact sensory nerves.

The effect of the combination of urea and urease on ethanol-induced gastric lesions and accompanying hyperemia in rats without or with the pretreatment with acetohydroxamic acid, a potent inhibitor of urease, is summarized in *Fig. 7*. Such pretreatment with acetohydroxamic acid (20 mg/kg i.g.) by itself neither affected the lesions provoked by 100% ethanol nor the fall in the GBF caused by this ulcerogen. When the acetohydroxamic acid was given together with the combination of urea and urease, the protective activity of urea and urease in ethanol-treated rats was completely abolished and this was accompanied by the drastic fall in the GBF similar to that recorded in control rats treated with 100% ethanol alone (*Fig. 7*).

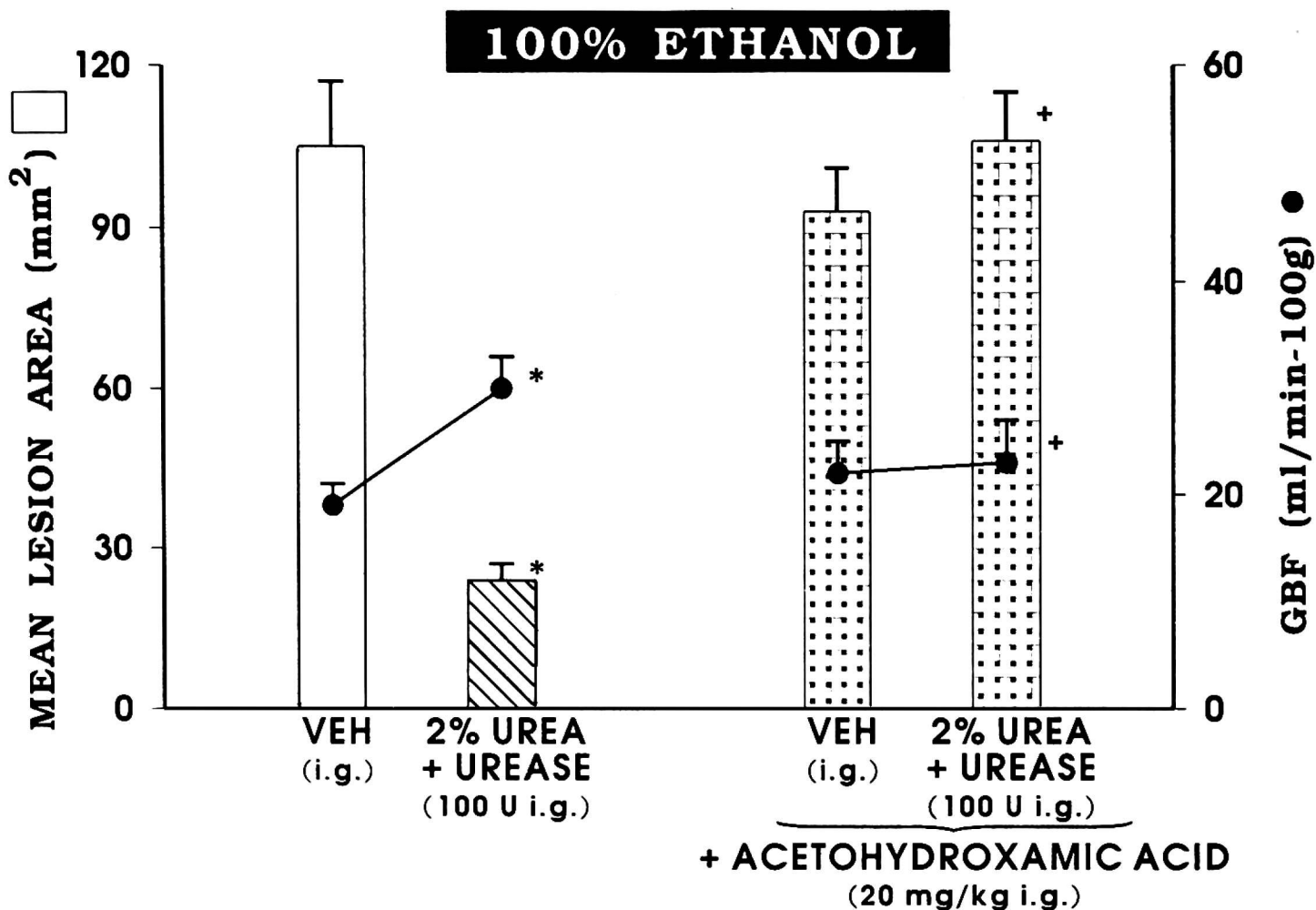


Fig. 7. Area of gastric lesions and changes in gastric blood flow induced by 100% ethanol in rats pretreated with vehicle or the combination of urea (2%) or urease (100 U) without or with addition of acetohydroxamic acid (20 mg/kg i.g.). Mean  $\pm$  SEM of 8–10 rats. Asterisk indicates significant change compared to vehicle control. Cross indicates significant change compared to the respective test without pretreatment with acetohydroxamic acid.

## DISCUSSION

This study demonstrates that  $\text{NH}_4\text{OH}$  applied i.g. in low concentration is highly effective in the protection of gastric mucosa against  $\text{NH}_4\text{OH}$  administered in highly damaging concentration. This protective activity of  $\text{NH}_4\text{OH}$  could be explained by the phenomenon of adaptive cytoprotection, that was previously described for certain “mild” irritants (19). The protection observed in our study with low concentration of  $\text{NH}_4\text{OH}$  was accompanied by the rise in the GBF and probably mediated by endogenous PG, NO-dependent pathway, histamine  $\text{H}_2$ -receptors and sensory nerves because it can be reversed by the pretreatment with indomethacin, L-NAME, ranitidine, and capsaicin-induced deactivation of sensory nerves. Furthermore, we demonstrated that  $\text{NH}_4\text{OH}$  produced by the hydrolysis of urea and urease exhibited mild irritant properties and greatly attenuated ethanol-induced gastric damage. This protection and accompanying hyperemia

induced by urea-urease administration were completely abolished by simultaneous exposure to acetohydroxamic acid, a potent inhibitor of urease activity.

Previous studies suggested that the infection of gastric mucosa with *Hp* and the presence of urease, especially in patients with peptic ulcer disease (24), may be an important source of a potent gastrotxin,  $\text{NH}_4\text{OH}$ . The significant correlation between the severity of gastric inflammation and the gastric juice concentration of  $\text{NH}_4\text{OH}$  supported the potential pathogenic role of ammonia in *Hp*-associated gastric injury. It has been suggested that  $\text{NH}_4\text{OH}$  generated in the gastric mucosa by the action of *Hp* urease enables to survive in gastric environment and facilitates this bacteria to colonize, thus predisposing the gastric mucosa to gastritis and of gastroduodenal ulcerations.

In this study, we tested the hypothesis that  $\text{NH}_4\text{OH}$  applied in low concentration acts as mild irritant and protects the gastric mucosa from the damage induced by high concentration of this agent through adaptive cytoprotection. This short-term adaptation of the gastric mucosa was originally proposed by Robert et al (25) and confirmed by our group, demonstrating highly protective activity of certain mild irritants (dilute ethanol, hyperosmolar solution or low concentrated bile salts) against the gross mucosal damage by strong irritants such as absolute ethanol. This short-term adaptation of the gastric mucosa was originally shown to be mediated by endogenous PG (24, 25), but more recently the role of PG has been questioned (26) and other mediators such as nitric oxide (19) or sensory nerves (27, 28) have been implicated in the adaptive cytoprotection in the gastric mucosa exposed to mild irritants.

$\text{NH}_4\text{OH}$  appears to induce adaptive cytoprotection that is mediated, at least in part, by endogenous PG because pretreatment with indomethacin in the dose capable of suppressing the mucosal generation of PG by over 80% attenuated the protection and accompanying rise in GBF induced by low concentration (15 mM) of  $\text{NH}_4\text{OH}$  applied before high concentration (250 mM) of this irritant. Another mediator of adaptive cytoprotection caused by low concentration of  $\text{NH}_4\text{OH}$  is probably NO, because the blockade of constitutive enzyme, NO synthase, by L-NAME, attenuated this protection and gastric hyperemia. This suggests, that NO may also contribute to adaptive cytoprotection afforded by low concentration of  $\text{NH}_4\text{OH}$ . The reduction in gastric protection and hyperemia elicited by L-NAME was reversed by co-administration of the substrate for NOS i.e. L-arginine, whereas its antiomer, D-arginine, which is not a substrate for NOS remained without effect. It is also possible that sensory nerves and neuropeptides released from the nerve endings such as calcitonin gene related peptide (CGRP) (22, 32) is involved in adaptive cytoprotection by  $\text{NH}_4\text{OH}$  because capsaicin-induced deactivation of sensory nerves greatly attenuated the protective and hyperemic responses accompanying this short-term adaptation.

An interesting finding of our present study is that blockade of H<sub>2</sub>-receptors by specific antagonist, ranitidine, which produced achlorhydria in the stomach almost completely reversed adaptive cytoprotection induced by small concentration of NH<sub>4</sub>OH. This could be explained that the elimination of acidic milieu in the gastric lumen prevented the conversion of NH<sub>4</sub>OH to NH<sub>4</sub>Cl leading to the aggravation of gastric lesions induced by higher concentration of NH<sub>4</sub>OH.

It is of interest that rats treated with NH<sub>4</sub>OH showed a significant increase in serum gastrin level and that increment was further augmented when the combination of 15 mM NH<sub>4</sub>OH plus 250 mM NH<sub>4</sub>OH particularly in rats pretreated with ranitidine to induce achlorhydria. The observed increments in plasma gastrin could be simply attributed to the neutralization of G-cells and prevention of acid inhibition of gastrin release due to *Hp*-associated generation of NH<sub>4</sub>OH in intral mucosa as suggested previously (29). Mechanism of the increase in serum gastrin level produced by NH<sub>4</sub>OH remains to be tested but our finding with ranitidine strongly suggests that gastrin-histamine link is probably involved in adaptive cytoprotection induced by NH<sub>4</sub>OH. This is in keeping with the hypothesis that hypergastrinemia associated with prolonged *Hp* infection contributes to increased parietal cell mass and the enhanced secretion of gastric acid in *Hp*-infected humans (29).

Adaptive cytoprotection can be also an explanation for the protective and possibly mild irritant action of NH<sub>4</sub>OH generated by the hydrolysis of urea in the presence of urease as demonstrated in our present study. Previous studies have shown that the concentration of urea lower than 0.5% together with 100 U urease produced histologic but not macroscopic gastric mucosal injury, whereas neither urea nor urease alone was effective (12). Moreover, NH<sub>4</sub>OH administered in the relatively lower concentration of about 15–30 mM failed to induce any gross gastric mucosal damage but when rats were subjected to ischemia gastric lesions were apparent probably due to the production of hypochlorous acid and monochloramines (5). In our present study, concurrent treatment with urea and urease greatly attenuated the formation of gastric lesions induced by ethanol and this was accompanied by the rise in the gastric microcirculation. This adaptive protective and hyperemic effects caused by urea and urease in the stomach were antagonized by pretreatment with indomethacin or L-NAME and significantly diminished following capsaicin induced deactivation of sensory nerves, thus emphasizing the role of endogenous PG, NO-arginine pathway and sensory nerves in the adaptation process.

Our data strongly suggests that urea-urease system may be rather beneficial for mucosal integrity presumably by producing NH<sub>4</sub>OH acting as a mild irritant and protecting the gastric mucosa from the damage induced by ethanol. This notion is corroborative with recent observation of Takeuchi et al (12) who found that measurable amount of NH<sub>4</sub>OH generated by the action of



urea-urease is effective in reducing lesions caused by topical application of acidified ethanol. In addition, we found that the protective and hyperemic effects of urea-urease system were completely eliminated by adding to this system of acetohydroxamic acid, a potent urease inhibitor (12). This finding indicates again that  $\text{NH}_4\text{OH}$  produced by hydrolizing urea with urease in the gastric lumen may be responsible for protective and hyperemic responses probably due to its mild irritant action on the gastric mucosa.

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