Irsogladine Prevents Monochloramine-Induced Gastric Mucosal Lesions by Improving the Decrease in Mucosal Blood Flow Due to the Disturbance of Nitric Oxide Synthesis in Rats

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Abstract. The inhibitory effect of an anti-ulcer drug irsogladine [2,4-diamino-6-(2,5-dichlorophenyl)-s-triazine maleate] on monochloramine (NH₂Cl)-induced gastric mucosal lesions and its mechanisms of action were clarified in rats. Irsogladine dose-dependently prevented the formation of gastric mucosal lesions induced by 60 mM NH₂Cl. The mucosal protective effect of irsogladine was not influenced by capsaicin-sensitive sensory defunctionalization. On the other hand, its protective effect was diminished by the inhibitor of nitric oxide synthase N⁵-nitro-L-arginine methylester (L-NAME), but not by the inducible nitric oxide synthase selective inhibitor aminoguanidine. Irsogladine restored the NH₂Cl-induced decrease in the gastric cGMP formation as an index of nitric oxide synthesis, while it alone had no influence on the cGMP formation in intact tissues. Pretreatment with L-NAME abolished the recovery of cGMP by irsogladine. Furthermore, irsogladine ameliorated the NH₂Cl-induced decrease in gastric mucosal blood flow, which was also reversed by pretreatment with L-NAME. These findings suggest that the improvement of the decrease in mucosal blood flow subsequent to the disturbance of gastric nitric oxide synthesis is involved in the protective effect of irsogladine on gastric mucosal lesions caused by NH₂Cl.

Keywords: irsogladine, monochloramine, nitric oxide, cyclic GMP, gastric mucosal blood flow

Introduction

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reported that either exogenous or endogenous NH₂Cl at much lower concentrations than ammonia produces gastric mucosal lesions in rats (4, 5).

Irsogladine (IG) [2,4-diamino-6-(2,5-dichlorophenyl)-s-triazine maleate], an antiulcer drug, has been shown to prevent gastric mucosal damages in several experimental animal models without inhibiting gastric secretion (6, 7). Although the mechanisms for the mucosal protective effect of IG have not been fully elucidated, IG has demonstrated to activate gap junctional intercellular communication and subsequently enhance gastric mucosal barrier functions by potentiating cellular integrity (8, 9). On the other hand, IG was also reported to inhibit the production of cytokines such as IL-8, IL-6, and RANTES in gastric epithelial cell lines infected with H. pylori in vitro (10). More recently, Yamamoto et al. (11) demonstrated that IG prevented the development of gastric mucosal damage induced by NH₂Cl, and this protective action of IG was significantly attenuated
by pretreatment with \( \text{N}^\text{G} \)-nitro-L-arginine methylester (L-NAME), suggesting that the protective action of IG may be mediated by endogenous nitric oxide (NO). However, their study provided no evidence about how the endogenous NO modulated by IG could be involved functionally with the mucosal protective factors.

In the present study, we found new evidence that the modulation of NO synthesis derived from constitutive NO synthase contributed to the gastroprotective effect of IG. The effect of IG on the \( \text{NH}_2\text{Cl} \)-induced decrease in gastric mucosal blood flow (GMBF) was subsequently investigated in relation to the changes of NO synthesis.

Materials and Methods

Animals

Male Sprague-Dawley rats, weighing 200 – 250 g (Charles River, Shizuoka), were used in all experiments. The animals were housed under standardized conditions for light and temperature and were fasted for 18 h prior to the experiments, but allowed free access to tap water. All experimental procedures described here were approved by the Committee for the Institutional Care and Use of Animals at Nippon Shinyaku Co. in accordance with the guidelines for animal experimentation. Chemical sensory deafferentation was induced by consecutive injections of capsaicin (20, 30, and 50 mg/kg, s.c.) for 3 days, 2 weeks before the experiment according to the method of Takeuchi et al (12). In this case, capsaicin was injected under ether anesthesia, and the rats were pretreated with terbutaline (0.1 mg/kg, i.m.) and aminophylline (10 mg/kg, i.m.) to counteract the respiratory impairment associated with capsaicin injection. On the day before the experiment, the effectiveness of the treatment was determined by evaluating the response to topical application of a capsaicin solution (0.1 mg/ml) in the eye. Capsaicin-treated animals that showed any unnatural wiping movements of the eye were excluded from the study.

Induction of gastric lesions by \( \text{NH}_2\text{Cl} \)

The solution of \( \text{NH}_2\text{Cl} \) was prepared according to the modified method of Thomas et al. (13). In brief, \( \text{NH}_2\text{Cl} \) was synthesized at 4 °C by adding NaOCl to ammonia solution in 10 mM phosphate buffer immediately before use (final pH 8.5). The concentration of \( \text{NH}_2\text{Cl} \) was determined assuming molar extinction coefficients of 429 at 242 nm.

The fasted rats were orally given 1 ml \( \text{NH}_2\text{Cl} \) at a concentration of 60 mM. Rats were killed by an overdose of ether 1 h after administration of \( \text{NH}_2\text{Cl} \). For macroscopic evaluation of the mucosal damage, the stomachs were excised and inflated by injection of 10 ml of 2% formalin. They were immersed in 2% formalin for 15 min to fix the gastric walls and opened along the greater curvature. The area of the each haemorrhagic lesion was measured under a dissecting microscope with a square grid (×10), summed, and used as a lesion index (mm²). IG or capsaicin was administered orally 30 min before \( \text{NH}_2\text{Cl} \) treatment. Amino-guanidine or ruthenium red was administered subcutaneously 60 min before \( \text{NH}_2\text{Cl} \) treatment. L-NAME was injected intravenously 10 min before oral administration of IG. L-Arginine was administered intraperitoneally 10 min before L-NAME injection.

Measurement of NO synthesis in gastric fundic tissues

The NO formation was estimated from cGMP content in rat stomachs, as described previously (14). Briefly, rats were killed by an overdose of ether 1 h after administration of 60 mM \( \text{NH}_2\text{Cl} \). The gastric fundic tissue was excised and homogenized with perchloric acid (final concentration was 0.2 M) and the homogenate centrifuged at 10,000 × g for 15 min at 4°C. The resultant supernatant was neutralized with 10% K₂CO₃ and then centrifuged again at 10,000 × g for 15 min at 4°C. The cGMP content in the supernatant was determined using a cGMP enzyme immunoassay kit (Amer sham Biosciences, Tokyo).

Measurement of GMBF

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and the trachea was cannulated to ensure a patent airway. The catheters were indwelled in the right and left femoral vein for intravenous administration of the test drug and continuous infusion of pentobarbital (10 mg/kg per hour), respectively. Another catheter was also indwelled in the duodenum for drug administration. The stomach was then exposed, mounted on the ex-vivo chamber, and perfused at a flow rate of 1 ml/min with saline during the test period. A heating pad kept the rectal temperature at about 37°C. The GMBF was measured by using a laser Doppler flowmeter (model ALF-21; Advance, Tokyo), placing the probe lightly on the fundic mucosa. This equipment provides no absolute values but does permit measurement of relative changes in GMBF. After the stability of GMBF was confirmed, the perfusion was interrupted and the solution in the chamber was withdrawn. The mucosa was then exposed to 2.5 ml of 60 mM \( \text{NH}_2\text{Cl} \) for 15 min. After exposure to \( \text{NH}_2\text{Cl} \), the mucosa was rinsed with saline several times and the perfusion with saline was resumed. Thereafter, GMBF was continuously recorded for another 60 min. In this case, IG was administered intraduodenally 30 min before \( \text{NH}_2\text{Cl} \) instillation in the chamber. L-
NAME was administered intravenously 10 min before IG treatment.

**Drugs**

IG was synthesized at Nippon Shinyaku Co., Ltd. (Kyoto). Capsaicin, ruthenium red, and L-arginine were purchased from Nacalai Tesque (Kyoto). L-NAME was from Dojindo (Kumamoto). Aminoguanidine, terbutaline, and aminophylline were from Sigma Chemicals (St. Louis, MO, USA). IG was suspended in 0.5% methylcellulose. Capsaicin was dissolved in saline containing 10% ethanol and 10% Tween 80 (w/w). Other drugs were dissolved in saline.

**Statistical analyses**

Statistical analyses were performed by the SAS program (SAS/STAT, version 8.02; SAS Institute, Inc., Cary, NC, USA). Data were analyzed for statistical significance by Dunnett’s or Tukey test for multiple comparison.

**Results**

**Effect of IG on NH₃Cl-induced gastric lesions**

Intragastric administration of NH₃Cl at a concentration of 60 mM produced severe hemorrhagic lesions in rat stomachs, the lesion score being 150.0 ± 24.7 mm² (Fig. 1). Oral administration of IG (0.3 – 3 mg/kg) dose-dependently prevented the formation of gastric lesions by NH₃Cl. As shown in Fig. 2, chemical ablation of capsaicin-sensitive sensory neurons aggravated the severity of NH₃Cl-induced gastric lesions. IG (3 mg/kg, p.o.) significantly prevented the formation of gastric lesions by NH₃Cl in both intact and sensory denervated rats. On the other hand, oral administration of capsaicin (20 mg/kg) prevented the NH₃Cl-induced gastric lesions in the intact rats, but was no longer effective in sensory denervated rats. Ruthenium red is known to be a blocker of capsaicin-activated cation channels (15, 16). As shown in Fig. 3, the protective action of IG was not affected by pretreatment of ruthenium red.

Prior administration of L-NAME (10 mg/kg, i.v.) significantly reversed the protective action of IG (3 mg/kg, p.o.) against NH₃Cl-induced gastric lesions, although L-NAME alone did not show any influence on the NH₃Cl-induced gastric lesions (Fig. 4). The inhibitory effect of L-NAME on the IG-induced mucosal protection was almost completely abolished by L-arginine (200 mg/kg, i.p.). In contrast, the protective action of IG against NH₃Cl-induced gastric lesions was not
affected by prior administration of aminoguanidine (10 mg/kg, s.c.), an inducible NO synthase selective inhibitor (Fig. 5).

**Effect of IG on cGMP content in NH$_2$Cl-induced damaged stomach**

To determine whether the NO formation is modulated by IG, the cGMP content, an index of NO synthesis, was measured in intact as well as in the NH$_2$Cl-treated stomachs in the absence or presence of IG. As shown in Fig. 6, the cGMP content was reduced in gastric fundic tissue at 1 h after 60 mM NH$_2$Cl treatment. IG (3 mg/kg, p.o.) restored the decrease of the cGMP content caused by NH$_2$Cl. This effect of IG was completely abolished by the prior administration of L-NAME (10 mg/kg, i.v.). It was notable that IG alone showed no effect on cGMP formation in intact stomachs.

**Effect of IG on GMBF responses to NH$_2$Cl**

Under pentobarbital-anesthetized conditions, the values for GMBF were relatively constant (approximately 40 ml·min$^{-1}$·100 g$^{-1}$, arbitrary unit) during a test period. Immediately after exposure to 60 mM NH$_2$Cl, the GMBF increased transiently, and the maximal responses reached 183.7 $\pm$ 35.0% of the basal value. Thereafter, GMBF was rapidly returned to basal level.
Discussion

IG inhibited the development of NH₄Cl-induced gastric mucosal lesions in a dose-dependent manner. The mucosal protective effect of IG was significantly attenuated by prior administration of L-NAME, a NO synthase inhibitor. This effect of L-NAME was antagonized by the pretreatment with L-arginine, a substrate of NO synthase. These findings indicate the involvement of endogenous NO in the protective action of IG on NH₄Cl-induced mucosal lesions, as was previously reported by Yamamoto et al. (11).

NO derived from constitutive NO synthase plays an important role in the gastrointestinal mucosal protection through regulating GMBF, acid, alkali, and mucus secretion (17, 18). It has also been reported that the activity of constitutive NO synthase is remarkably reduced in rat gastric damaged mucosa caused by water immersion restraint stress or by treatment of indomethacin (19, 20). In contrast, a large amount of NO liberated by inducible NO synthase contributes to the inflammatory response in experimentally induced gastrointestinal lesions (21–23). That is to say, a dual role of endogenous NO, defensive and detrimental on the gastrointestinal mucosa, has been demonstrated. In the present study, the mucosal protective effect of IG against NH₄Cl-induced lesions was not affected by a selective inducible NO synthase inhibitor, aminoguanidine, ruling out the possibility that the inducible NO synthase is involved in the protective effect of IG on the NH₄Cl-induced mucosal lesions.

In the present study, NO synthesis was estimated from cGMP content, since NO stimulates soluble guanylate cyclase to produce cGMP in a variety of tissues (14, 24, 25). The cGMP content markedly decreased in NH₄Cl-induced damaged stomach, indicating the reduction of NO synthase activity by NH₄Cl. IG reversed the decrease in cGMP content induced by NH₄Cl, and pretreatment with L-NAME abolished this inhibitory effect of IG. Moreover, it is noteworthy that there is a good correlation between the protective effect of IG against NH₄Cl-induced mucosal lesions and its restoration of NO synthesis. Taken together, it is suggested that the modulation of the activity of constitutive NO synthase contributes at least in part to the gastroprotective effect of IG. This is highly important new evidence found in this study.

At present, we do not know in detail the mechanisms underlying the modulatory effect of IG on gastric NO synthesis. Since IG alone did not manifest any marked effect on cGMP formation in normal gastric tissue in vivo, it seems likely that the protective effect of IG is mediated by the maintenance of constitutive NO synthase, rather than direct activation of NO synthase enzyme. In fact, IG affected neither the activity of constitutive NO synthase nor that of inducible NO synthase (T. Kyo et al., unpublished observations). In an inflammatory condition in the gastrointestinal tract, a large amount of superoxide anion (O₂⁻) is generated through the NADPH oxidase of infiltrating neutrophils and macrophages or tissue xanthine oxidase. O₂⁻ is counteracted by superoxide dismutase, an endogenous anti-oxidative agent. Meanwhile, O₂⁻ reacts with NO to produce highly cytotoxic peroxynitrite, a primary pathway of NO metabolism (26). It may be likely that irsogladine suppresses O₂⁻ production, followed by inhibiting the formation of peroxynitrite and NO reduction.

It has been shown that capsaicin sensitive sensory nerves are abundantly distributed throughout the gastric mucosa and play an important role in maintaining the homeostasis of gastric mucosa (27). These capsaicin sensitive sensory nerves exert the protective action through calcitonin gene related peptide that increases...
GMBF by inducing NO formation in the vascular endothelium (28). It has also been reported that NH$_2$Cl impairs the gastric hyperemic response by the dysfunction of capsaicin-sensitive sensory nerves, which leads to the retardation of the recovery or healing of gastric lesions (29, 30). However, in the present study, the protective effect of IG against NH$_2$Cl-induced lesions was not affected by denervation of capsaicin-sensitive sensory nerves or pretreatment with ruthenium red, a blocker of capsaicin-activated cation channels. Therefore, capsaicin-sensitive sensory nerves are not likely to be involved in the mucosal protective effect of IG.

With regards to the NO-derived physiological functions in gastric mucosa, regulation of GMBF by NO has been attracting particularly close attention. GMBF plays very important roles in adaptive responses against the mucosal injury induced by noxious agents such as acid and in the healing process of damaged mucosa (28, 31). It has also been reported that the treatment with indomethacin or HCl-aspirin reduced GMBF in rats (32, 33). In the present study, we confirmed that NH$_2$Cl caused a transient increase followed by a prolonged decrease in GMBF. To clarify the interrelationship among the mucosal protective effects of IG, endogenous NO and GMBF, the effect of IG on the decrease in GMBF induced by NH$_2$Cl was investigated. IG restored the decrease of GMBF induced by NH$_2$Cl and this effect was obviously attenuated by pretreatment with L-NAME. IG neither affected the gastric NO formation in normal rats nor had any influence on the normal GMBF.

Ueda et al. (34) reported previously that IG alone had almost no effect on GMBF in anesthetized dogs, but it produced a marked increase of GMBF when GMBF was decreased by treatment with indomethacin. These results suggest that IG causes the improvement of GMBF by modulating endogenous NO, in particular, under the conditions that GMBF is reduced by ulcerogenic stimuli such as noxious agents. This is the first report that an anti-ulcer drug recovered the decrease of GMBF induced by NH$_2$Cl treatment, resulting in the protection against gastric mucosal damages. The present study suggests that IG may have therapeutic potential in the prevention of _H. pylori-related_ gastritis.

In conclusion, the improvement of GMBF due to the modulation of NO generated by constitutive NO synthase contributes to the protective effect of IG against NH$_2$Cl-induced gastric mucosal injury.

References


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