Role of gap junctions in inhibiting ischemia-reperfusion injury of rat gastric mucosa

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1Department of Internal Medicine, NTT Tokai General Hospital, Nagoya 460-0017; 2First Department of Internal Medicine, Nagoya City University School of Medicine, Nagoya 467-0001; and 3Discovery Research Laboratories II, Nippon Shinyaku Co. Ltd., Kyoto 601-8550, Japan

Iwata, Fumihiro, Takashi Joh, Fusao Ueda, Yoshihiro Yokoyama, and Makoto Itoh. Role of gap junctions in inhibiting ischemia-reperfusion injury of rat gastric mucosa. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G883–G888, 1998.—Gap junctional intercellular communication (GJIC) is known to be important in the maintenance of tissue homeostasis. However, the role of GJIC in gastric mucosa has not been well investigated. We tested the hypothesis that maintenance of GJIC protects rat gastric mucosa against ischemia-reperfusion (I/R) stress by using irsogladine, an activator of GJIC, and octanol, an inhibitor of GJIC. Intragastri- perfusion with octanol before ischemia resulted in a significant increase in 51Cr-EDTA clearance after reperfusion. Intraduodenal pretreatment with irsogladine attenuated the increase in 51Cr-EDTA clearance produced by octanol in a dose-dependent manner. Epithelial gap junctions reacted with anticonnexin-32 monoclonal antibodies were not changed after I/R stress alone. Intragastric perfusion with octanol caused a significant reduction in immunoreactive connexin-32 spots, which was completely reversed by irsogladine. These results indicate that inhibition of GJIC weakens the barrier function of gastric mucosa and subsequently causes damage of the barrier function in combination with I/R. Facilitation of GJIC and maintenance of gap junctions protect gastric mucosal barrier functions by potentiating cellular integrity.

Gap junctional intercellular communication; free radicals; 51Cr-labeled ethylenediaminetetraacetic acid clearance; irsogladine; octanol

THE INDIVIDUAL CELLS that compose tissues do not function independently of neighboring cells, and the function of each cell is regulated by a homeostasis network. The function of gastrointestinal epithelial cells has been attributed to junctional structures, including tight junctions, adherens junctions, desmosomes, gap junctions, and interdigitations. Each junctional structure regulates the function of other junctional structures.

Gap junctions are composed of connexons, which are membrane protein oligomers of adjacent cells in direct contact with each other (9), and are permeable to low-molecular substances, including ions, nutrients, metabolites, and second messengers (1, 20, 34a). The connexon is a cylindrical assembly of six connexins, which are identical rod-shaped monomers (8, 21). Gap junctional intercellular communication (GJIC) regulates the functions of multicellular systems composed of homologous and heterologous cells.

Gap junctions of gastric surface mucous cells have been shown to be disrupted by water-immersion stress before mucosal damage, which additionally requires luminal acid (32). In addition, the inhibition of GJIC by octanol through permeation into the lipid bilayer near gap junctions accelerates stress ulceration (32). These findings suggest that cellular integrity of gastric surface mucous cells mediated by GJIC is responsible for the primary defense of gastric mucosa by preventing penetration of luminal acid and exogenous noxious agents to the serosa under stress conditions. A decrease in the effectiveness of gap junctions has been postulated to be associated with gastric ulcer relapse in patients (23). A typical inhibitor of GJIC, 12-O-tetradecanoylphorbol 13-acetate, is well known to be a damaging agent to colonic mucosa (7). These facts suggest an important role for gap junctions in protecting gastrointestinal mucosa.

In a model of restricted ischemia-reperfusion injury induced by clamping the left gastric artery, no gross morphological lesions are observed and the number of microscopic lesions is too small for quantification (17). 51Cr-labeled EDTA clearance, which was originally developed to assess mucosal blood-to-lumen permeability (2, 3, 13), allows assessment of such limited morphological damage induced by restricted ischemia-reperfusion (17). Thus induced mucosal damage is considered to be due to functional disruption of the epithelial lining.

Although there is accumulating evidence (10, 12, 37) that oxygen radicals play a role in the pathogenesis of cell and tissue injuries induced by ischemia and subsequent reperfusion, the mechanism of ischemia-reperfusion-induced injury is completely unknown. Oxidative stress has been shown to alter normal expression of connexins in hepatocytes (18). Therefore, functional disruption of epithelium caused by ischemia-reperfusion may be derived from oxidative stress against gap junctions.

The objective of this study was to test the hypothesis that the gastric mucosal functional damage induced by restricted ischemia-reperfusion is caused by oxidative stress against gap junctions. In this study, octanol, which inhibits GJIC (4, 5, 14), and irsogladine, which activates GJIC through activation of the M1 muscarinic ACh receptor (29–31), were used to examine the effect on changes in 51Cr-EDTA clearance and epithelial immunoreactive gap junctions induced by ischemia-reperfusion.

MATERIALS AND METHODS

Animal Preparation for 51Cr-EDTA Clearance Measurement

Male Sprague-Dawley rats (250–350 g) were fasted overnight and anesthetized with intraperitoneal urethan (0.6
g/kg; Tokyo Kasei Kogyo, Tokyo, Japan) and α-chloralose (0.12 g/kg; Wako Pure Chemical Industries, Osaka, Japan). A tracheotomy was performed, and polyethylene tubing (PE-240) was inserted into the trachea to maintain an open airway. The right femoral artery and vein were cannulated (PE-50) to monitor systemic arterial pressure and administer 51Cr-EDTA, respectively. Body temperature was maintained at 36–38°C with a heating pad and lamp throughout the experiment.

Assessment of Mucosal Damage (Measurement of 51Cr-EDTA Clearance)

After an abdominal incision, the renal blood vessels were ligated to prevent loss of the low-molecular-weight tracer used to assess gastric mucosal permeability. The stomach was exteriorized, and a ligature was placed around the pylorus. After the incision of the forestomach, a double-lumen cannula (outer polyethylene tube, 3.25-mm diameter; inner polyethylene tube, 1-mm diameter) was inserted into the stomach and exteriorized, and a ligature was placed around the pylorus. The stomach was perfused with saline or solution containing octanol or ethanol through the inner cannula at a rate of 1.0 ml/min, and the perfusate was irrigated with normal saline and covered with plastic wrap to prevent evaporative fluid loss. After surgery, intravenous 51Cr-EDTA (3.7 MBq) was administered as a bolus. While perfusing the gastric lumen with saline, we continuously monitored gastric mucosal permeability by measuring 51Cr-EDTA clearance from blood to gastric lumen under control conditions, during ischemia, and after reperfusion. Once steady-state clearances were obtained (20–30 min), an additional 30-min perfusion served as a control. Subsequently, gastric ischemia was induced by clamping the left gastric artery for 20 min. After reperfusion, mucosal permeability was monitored for another 50 min.

Immunohistochemical Examination of Gastric Mucosal Gap Juncions

Production and characterization of monoclonal antibody (MAB) 6-3G11 have been described previously (27). This MAB is directed against the major rat liver gap junction protein, recognizing an epitope on the cytoplasmic side.

Preparation of Tissue Specimens

After removal, each stomach was placed in a condom and immersed quickly in liquid nitrogen. The frozen stomachs were embedded in Tissue-Tek (OCT Compound, Sakura Finetek, Torrance, CA) and stored at −80°C until use.

Study Design

Study 1: Effect of GJIC inhibitor without ischemia-reperfusion. To study the effects of octanol without ischemia-reperfusion, the rats received intragastric (IG) perfusion with 1% octanol for 30 min, followed by saline for 70 min. 51Cr-EDTA clearance was measured as described above.

Study 2: Effect of GJIC inhibitor on ischemia-reperfusion-induced gastric injury. Before the 20-min period of ischemia, the rats were divided into three groups that received IG perfusion with saline (control), 1% ethanol (5), or 1% octanol for 30 min. At the beginning of the ischemic period, the IG perfusate in all three groups was changed to saline until the end of the experiment. Systemic arterial pressure and 51Cr-EDTA clearance were measured as described above.

Study 3: Effect of GJ IC activator on octanol- and ischemia-reperfusion-induced gastric injury. To study the effects of irsogladine, an activator of GJIC, we assessed mucosal damage by measurement of 51Cr-EDTA clearance. Thirty minutes before the beginning of 51Cr-EDTA clearance measurements, the rats were divided into four groups that received intraduodenal vehicle (5 ml/kg 0.5% methylcellulose) or irsogladine (3, 10, or 30 mg/kg). Before the 20-min period of ischemia, all of the rats received IG perfusion with 1% octanol for 30 min. The IG perfusate was changed to saline at the beginning of the ischemic period, and saline was maintained for the rest of the experiment. Systemic arterial pressure and 51Cr-EDTA clearance were measured as described above.

Study 4: Immunohistochemistry. Study Changes in gap junctions were examined immunohistochemically as described previously (20). Animal preparation was the same as for 51Cr-EDTA clearance. The rats were divided into four groups that received a 30-min IG perfusion with saline, 1% ethanol, or 1% octanol or pretreatment with 30 mg/kg of irsogladine followed by a 30-min IG perfusion with 1% octanol. After the 30-min IG perfusion, 20 min of ischemia followed. The IG perfusate was changed to saline at the beginning of ischemia, and saline was maintained until the end of ischemia. Just after the end of ischemia, the stomachs were removed. Normal rat stomachs were removed for reference staining.

Frozen tissue sections taken from along the circular muscles of the fundus were cut to a thickness of 6 µm in a cryostat. The sections were incubated for 1 h at room temperature with 100 µg/ml of MAB 6-3G11. The sections were rinsed three times with 10 mM PBS (pH 7.4) for 5 min each. Secondary FITC-conjugated rabbit anti-mouse IgG (heavy and light chain) antibody at a 1:200 dilution in PBS was applied, and the sections were incubated for 1 h at room temperature. After three 5-min rinses with PBS, the sections were mounted in 60% glycerol in PBS and viewed under an Olympus fluorescence microscope. Specimens used to determine the background staining were treated in the same way, except for the application of primary antibody.

In each of the above-described studies, the treatments were administered in a randomized fashion. The randomization scheme was prepared by an assistant who did not know the result of the studies. The studies were approved by the Animal Research Committee of the Nagoya City University Medical School.

Statistical Analysis

ANOVA and Scheffé’s post hoc test for multiple comparisons were used to compare the mean values among the groups. P < 0.05 was considered significant. All data are expressed as means ± SE.
RESULTS

Study 1: Effect of GJIC Inhibitor Without Ischemia-Reperfusion

Luminal perfusion of 1% octanol without ischemia-reperfusion did not increase $^{51}$Cr-EDTA clearance (Fig. 1).

Study 2: Effect of GJIC Inhibitor on Ischemia-Reperfusion-Induced Gastric Injury

There was no significant difference in mean blood pressure among the groups (data not shown). Figure 2 shows the effects of luminal perfusion with ethanol or octanol on gastric mucosal permeability without ischemia-reperfusion in rats. Values represent means ± SE of 6 rats. In rats treated with octanol without ischemia-reperfusion, $^{51}$Cr-labeled EDTA clearance did not increase during the experiment. IG, intragastric.

Study 3: Effect of GJIC Activator on Octanol and Ischemia-Reperfusion-Induced Gastric Injury

There was no significant difference in mean blood pressure among these four groups (data not shown). Figure 3 shows the effects of irsogladine on gastric mucosal damage induced by IG octanol and 20 min of ischemia-reperfusion stress. In the saline-treated group, 20 min of ischemia resulted in a small increase in $^{51}$Cr-EDTA clearance after reperfusion (Fig. 2A). There was no significant increase in $^{51}$Cr-EDTA clearance during reperfusion in the ethanol-treated group compared with in the saline-treated group (Fig. 2B). Treatment with octanol significantly increased $^{51}$Cr-EDTA clearance during reperfusion (Fig. 2C).

Fig. 1. Effects of luminal perfusion with octanol on gastric mucosal permeability without ischemia-reperfusion in rats. Values represent means ± SE of 6 rats. In rats treated with octanol without ischemia-reperfusion, $^{51}$Cr-labeled EDTA clearance did not increase during the experiment.

Fig. 2. Effects of luminal perfusion with ethanol or octanol on the increase in gastric mucosal permeability induced by ischemia-reperfusion in rats. Values are means ± SE of 6 rats. In rats treated with saline (A) or ethanol (B), $^{51}$Cr-EDTA clearance did not increase during period of ischemia and increased slightly after reperfusion. Treatment with octanol (C) significantly increased $^{51}$Cr-EDTA clearance after reperfusion. *P < 0.05 compared with values in A. †P < 0.05 compared with values in B.

Fig. 3. Effect of irsogladine on the increase in gastric mucosal permeability induced by the combination of luminal perfusion with octanol and ischemia-reperfusion in rats. Values are means ± SE of 6 rats. Irsogladine (3-30 mg/kg) dose dependently inhibited the increase in $^{51}$Cr-EDTA clearance induced by the combination of octanol perfusion and ischemia-reperfusion. *P < 0.05 compared with values in A.
were significant decreases in $^{51}$Cr-EDTA clearance during reperfusion in the 10 and 30 mg/kg irsogladine-pretreated groups compared with in the vehicle-pretreated groups (Fig. 3, C and D).

### Study 4: Immunohistochemical Study

Immunoreactive areas in the surface mucous cells appeared as a stringlike lining between lateral membranes (Fig. 4A). The combination of a 30-min gastric perfusion with saline or ethanol and 20 min of ischemia did not cause any change in immunoreactive gap junctions in surface mucous cells at the end of ischemia (Fig. 4, B and C). The combination of a 30-min perfusion with octanol and 20 min of ischemia changed the shape of the immunoreactive spots (Fig. 4D), whereas octanol perfusion without ischemia did not change immunoreactive gap junctions (data not shown). Pretreatment with 30 mg/kg irsogladine prevented the changes in immunoreactive gap junctions induced by the combination of octanol perfusion and ischemia (Fig. 4E).

### DISCUSSION

Junctional structures, including adherens junctions (22), tight junctions (28), and gap junctions (1), are now known to regulate many cellular functions. Gastric gap junctions are numerous in the luminal surface (16), and the size of gap junction develops during the maturation of surface mucous cells (19), while intestinal gap junctions are present in crypt cells but not in mature villus cells in rats and mice (15). In cultured rabbit gastric epithelial cells, GJIC has been shown to be facilitated by irsogladine and dibutyryl-cAMP (31), both of which protect gastric mucosa (33). These facts suggest that cellular integrity of gastric epithelial cells mediated by GJIC is responsible for the primary defense of the gastric mucosa against noxious luminal agents. However, it has also been found that disruption of gap junctions alone does not cause gastric mucosal damage under stress conditions. Additional stimuli, especially luminal acid, applied to gastric mucosa with disrupted gap junctions cause mucosal damage (30). Therefore,
gap junctions, by potentiating the integrity of the epithelial lining may prevent at least lumen-to-serosa permeation of luminal acid and other noxious agents. Indeed, isoglutamine inhibited lumen-to-serosa permeation of noxious ethanol and aspirin in rats (34).

The $^{51}$Cr-EDTA clearance measurement technique was developed to assess mucosal blood-to-lumen permeability and has been shown to be an extremely sensitive index of functional mucosal damage (2, 3, 13, 17). Because a 30-min ischemic period causes too great an increase in $^{51}$Cr-EDTA clearance (17), we chose a 20-min ischemic period in studies 1 and 2. A decrease in mean blood pressure could affect mucosal permeability in $^{51}$Cr-EDTA clearance measurements, but there was no significant difference in mean blood pressure between the control group and the other groups.

Octanol, but not ethanol, significantly aggravated ischemia-reperfusion-induced gastric mucosal injury concomitantly with changes in the location and shape of immunoreactive areas associated with gap junction proteins. This mucosal injury was caused immediately after reperfusion, reaching a peak 10 min after and recovering near basal levels within 100 min. Thus the increase in $^{51}$Cr-EDTA clearance induced by luminal octanol perfusion and ischemia-reperfusion was considered to be a functional and reversible phasic response. The gap junctions located in the adjacent cell membranes, which were stringlike immunoreactive spots, may have the ability to communicate functionally with neighboring cells. In contrast, a lumpy immunoreactive appearance of gap junction proteins, which may represent annular gap junctions, is considered to reflect a loss of communication ability. Therefore, octanol- and ischemia-induced changes in the location and shape of immunoreactive gap junctions may decrease GJIC ability and disturb cellular integrity. These results indicate that impairment of GJIC function by octanol may induce some pathogenic changes related to ischemia-reperfusion injury within 10 min after reperfusion.

Pretreatment with isoglutamine inhibited in a dose-dependent fashion both the increase in $^{51}$Cr-EDTA clearance and the changes in location and shape of immunoreactive gap junctions induced by the combination of octanol and ischemia-reperfusion. These results show that functional gap junctions are critical to maintaining the function of epithelial lining.

In the present study, the combination of octanol infusion and ischemia before reperfusion did not increase $^{51}$Cr-EDTA clearance, despite changes in immunoreactive gap junctions that had already occurred; the increase in $^{51}$Cr-EDTA clearance occurred 10 min after reperfusion. A similar phenomenon has been observed (32) under water-immersion stress conditions; disruption of gap junctions per se does not facilitate stress ulceration in the absence of additional noxious stimuli. Thus GJIC is considered to be one of the underlying defense factors of gastric mucosa. Therefore, the weakening of epithelial integrity caused by octanol may be further aggravated by ischemia-reperfusion. Finally, gap junctions may maintain the blood-to-lumen barrier function in addition to lumen-to-serosa barrier functions already reported (34).

Changes in gap junctions have been demonstrated in Alzheimer’s disease (35), ischemic injury of the brain (6), heart disease (26), liver injury (25), vascular diseases (24), and carcinogenesis (36). Given the results in the present study, disorders of gastric mucosa may also be associated with disrupted gap junctions.

In conclusion, these findings suggest that GJIC acts as an important protective factor against ischemia-reperfusion stress in rat gastric mucosa and that disruption of gap junctions may be one of the causal factors in ischemia-reperfusion injury of rat gastric mucosa.

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Received 24 December 1997; accepted in final form 15 June 1998.

REFERENCES

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