Ischemic preconditioning is considered as the most powerful gastroprotective intervention against mucosal lesions and ulcers, but the mechanism of this phenomenon has been little examined. In this study, we tested the effects of inactivation of sensory nerves in a new rat model combining acute gastric erosions with subsequent ulcers induced by ischemia-reperfusion (I/R). I/R lesions were produced in rats by clamping the celiac artery for 0.5 h followed by 3 h of reperfusion in rats with intact or inactivated sensory nerves by pretreatment with capsaicin for two weeks before the I/R. The animals were killed at 0 and 3 h and 3 days after I/R and the area of gastric lesions was determined planimetrically, the gastric blood flow (GBF) by H2-gas clearance technique, and the plasma levels of gastrin by RIA. Gastric mucosal content of calcitonin gene-related peptide (CGRP) was assessed by RIA. Following I/R, gastric erosive lesions occurred after 3 h and these erosive lesions then progressed into gastric ulcers within 3 days in all rats. Sensory-inactivation with capsaicin caused a several fold increase in the area of early (at 3 h) acute lesions and later (at 3 d) gastric ulcers induced by I/R. This enhancement of acute and then chronic gastric lesions was accompanied by a significant fall in GBF, an elevation of plasma gastrin, and a decrease in mucosal expression of CGRP. Ischemic preconditioning markedly reduced acute lesions and chronic ulcerations induced by I/R and attenuated the changes in plasma gastrin and mucosal CGRP contents but these effects were significantly more pronounced in rats with intact sensory nerves but less in capsaicin-inactivated animals. We conclude that: 1) The I/R resulted in the formation of early acute gastric lesions followed 3 days later by chronic gastric ulcers and this gastric injury was accompanied by an impairment of gastric microcirculation, hypergastrinemia, and suppression of the gastric mucosal CGRP; 2) Gastric ischemic preconditioning significantly attenuated both acute mucosal damage and chronic ulcers induced by I/R, and this was accompanied by a rise in gastric blood flow; 3) The inactivation of sensory nerves with capsaicin enhanced the formation of I/R-induced acute and chronic gastric lesions and strongly attenuated the gastroprotection afforded by I/R, possibly due to the decline in mucosal blood flow and the fall in expression of integrity peptides such as CGRP; and 4) The excessive release of gastrin may limit the extent of mucosal lesions observed during progression of gastric erosions into ulcers induced by I/R.
KEY WORDS: Gastric preconditioning, ischemia-reperfusion, gastric blood flow, sensory nerves, calcitonin gene related peptide, gastrin

INTRODUCTION

Ischemia preconditioning is known to enhance the mucosal resistance to the deleterious effect of prolonged severe ischemia by previous exposures to brief vascular occlusions (1). These protective effects of ischemic preconditioning were first described in the heart by Murry and coworkers (2). Since that time, ischemic preconditioning has been shown to reduce the extent of myocardial infarct size as well as the damage to the brain, liver, kidneys, skeletal muscle and the stomach induced by exposure to severe ischemia with subsequent reperfusion in a variety of species (3—7), but the mechanism of this organ protection by such preconditioning has not been fully clarified.

The protective activity of ischemic preconditioning is best documented in the heart where short episodes of coronary occlusion were shown to prevent lethal injury of the myocardium induced by long-term and severe ischemia/reperfusion (I/R) (8, 9). In another report, ischemic preconditioning of rat mesenteric venules led to enhanced bioavailability of nitric oxide (NO) and abolished oxidant production resulting in the decrease in the leukocyte adhesion and emigration through mesentery (10). In our previous study, ischemic preconditioning was as effective in the gastroprotection against the damage caused by I/R and corrosive substances as the topical application of “mild irritants” (7) that are known to depend upon intact afferent sensory nerves (11, 12). This indicates that ischemic preconditioning requires intact sensory nerves, possibly providing excessive amounts of gastro-protective calcitonin gene related peptide (CGRP) in the gut (10, 13, 14). The question remains whether the protective influence of ischemic preconditioning against gastric damage induced by I/R concerns only acute post I/R lesions and whether it is also extended to chronic gastric ulcerations developing 2—3 days after the gastric mucosa was subjected to I/R. As the sensory nerves and CGRP have been implicated in the gastroprotection against acid-independent and acid-dependent gastroprotection (11, 15—19) it seems reasonable to predict that also ischemic preconditioning depends upon these factors.

The oldest mediators of gastroprotection, the prostaglandin (PG), were found to originate from at least two cyclooxygenases (COX), one constitutive (cyclooxygenase-1) playing physiological role in mucosal homeostasis and another, inducible (cyclooxygenase-2) isoform that is expressed at a site of inflammation (20, 21), and that has been found recently to be involved in gastric preconditioning (7).

This study was designed to confirm whether ischemic preconditioning exists in the stomach and, if so, whether it extends to the formation of chronic gastric
ulcers that follow acute post I/R damage and to elucidate the contribution of sensory nerves and mucosal integrity peptides such as CGRP and gastrin to the gastroprotection afforded by standard ischemic preconditioning.

MATERIAL AND METHODS

Male Wistar rats weighing 180—220 g were used in all studies. Rats were fasted 18 h before the experiment but they had free access to the drinking water. Studies were approved by Ethical Committee for Animal Research of Jagiellonian University.

Production of acute gastric lesions and chronic gastric ulcers induced by ischemia-reperfusion (I/R)

I/R-induced erosions were produced in 80 rats by the method originally proposed by Wada et al (22). Briefly, under pentobarbital anesthesia (50 mg/kg i.p.), the abdomen was opened, the celiac artery identified and clamped with a small device for 30 min followed by removal of the clamp to obtain reperfusion. In addition, short ischemia (occlusion of celiac artery 2 times for 5 min-ischemic preconditioning) was applied 30 min before subsequent exposure to longer (regular) 30 min ischemia (also induced by clamping of celiac artery) and followed by 3 h of reperfusion. The respective control group included the sham-operated control animals, whose celiac artery was only slightly manipulated but not occluded. First, we attempted to confirm the effect of gastric ischemic preconditioning on the acute gastric lesions induced by regular I/R. For this purpose, rats were preconditioned with two episodes of gastric preconditioning, each lasting 5 min, before the exposure to 30 min of ischemia followed by 3 h of reperfusion. Second, we determined the effects of ischemic preconditioning on the development of chronic gastric ulcerations which are known to reach maximum after 3 days upon the I/R (23).

The area of gastric lesions was determined using a planimetry (Morphomat, Carl Zeiss, Berlin, Germany) under blinded conditions according to the method described previously (24).

Implication of sensory afferent nerves and CGRP in gastric ischemic preconditioning

In tests with involvement of sensory nerves and neuropeptides in gastroprotection induced by gastric preconditioning, rats with capsaicin-induced deactivation of these nerves or those pretreated with calcitonin gene related peptide (CGRP) (23, 25, 26) were used. Role of sensory afferent nerves in
The protective effects of ischemic preconditioning was tested in rats with capsaicin-induced deactivation of these nerves. For this purpose the animals were pretreated with capsaicin (Sigma Co., St. Louis, MO) injected s.c. for 3 consecutive days at a dose of 25, 50 and 50 mg/kg about 2 weeks before the start of experiment as described previously (27). All injections of capsaicin were performed under ether anesthesia to counter the pain reactions and respiratory impairment associated with injection of this agent. To check the effectiveness of the capsaicin denervation, a drop of 0.1 mg/ml solution of capsaicin was instilled into the eye of each rat and the protective movements were counted as described previously (12). Control rats received injections with vehicle (saline). All animals pretreated with capsaicin showed negative wiping movement test, thus confirming functional denervation of the capsaicin-sensitive nerves. Standard ischemic preconditioning (2 × 5 min) was induced in rats with intact or capsaicin deactivated nerves and this was followed by I/R according to the procedure described above. In some group of capsaicin-denervated rats, CGRP (10 µg/kg) was applied s.c. 30 min before standard preconditioning followed by exposure to I/R, in order to check, whether deficiency of endogenou CGRP due to functional ablation of sensory nerves could be abolished by administration of exogenous neuropeptide at a dose that was reported to reverse the effects of capsaicin deactivation on gastric mucosa (12). The experimental protocol included the following study groups, each consisting of 6-8 animals; 1) vehicle (saline) followed 30 min later by standard preconditioning in rats with intact afferent nerves; 2) standard preconditioning followed 30 min later by I/R in rats with intact sensory nerves and killed after 3 h post-I/R; 3) standard preconditioning followed 30 min later by I/R in rats with intact sensory nerves and killing of animals after 3 days post-I/R; 4) vehicle (saline) followed 30 min later by I/R in rats with capsaicin-deactivated afferent nerves. In all series of experiments the area of lesions and the gastric blood flow were measured in similar manner as mentioned above.

**Measurement of gastric blood flow (GBF)**

At the termination of each experiment, the gastric blood flow (GBF) was measured by H₂-gas clearance technique. Rats were lightly anesthetized with ether, the abdomen was opened and the stomach was exposed. The gastric blood flow was measured in the oxyntic gland area of the stomach by means of local H₂-gas clearance method using an electrolytic regional blood flow meter (Biomedical Science, Model RBF-2, Japan) as described previously (23). The measurements were calculated in three areas of the mucosa and the mean absolute values (ml/100 g-min) of these measurements were calculated and
expressed as percent changes from those recorded in control animals treated with vehicle.

Determination of calcitonin gene-related peptide (CGRP) by RIA

Gastric mucosal biopsy samples (about 50 mg) were taken from the fundic mucosa immediately after anesthesia and the measurement of the lesion area and blood flow. The samples were weighted and placed in liquid nitrogen. Then they were homogenized and the CGRP was determined using CGRP (rat) RIA kit supplied by Phoenix Pharmaceutical, Inc, Belmont, CA in accordance with the manufacturer’s instruction. The antiserum used did not cross-reacted with any GI peptide except human CGRP. The sensitivity of the RIA was 32 pg/tube. The interassay and intraassay variability of these measurements were 4.5 and 2.9%, respectively.

Plasma gastrin in the blood withdrawn from the vena cava immediately after anesthesia was measured using RIA with antiserum (kindly provided by professor J. Rehfeld, Copenhagen, Denmark) that recognizes equally alpha-amidated gastrin-17 (G-17) and G-34 and used in final dilution of 1:500000. The sensitivity of the gastrin RIA was about 5 pg/tube and the interassay and intraassay variations were 12% and 8%, respectively.

Statistical analysis

Results are expressed as means ± SEM. The significance of the difference between means was evaluated using analysis of variance followed by Duncan’s test with a level of confidence at $P < 0.05$.

RESULTS

Effect of short ischemia on the acute gastric lesions and chronic gastric ulcers induced by ischemia/reperfusion insult and the accompanying changes in the GBF

Fig. 1 shows the effects of 30 min ischemia followed by 3 h reperfusion on the area of gastric mucosal damage in rats without and with double short (5 min) episodes of ischemia on acute gastric lesions and accompanying changes in the gastric blood flow. I/R alone caused the appearance of acute gastric lesions with an area amounting to about 100 mm² and chronic ulcers with an area averaging about 53 mm². Short ischemic episodes (5 min each), caused reduction in the areas of acute I/R lesions monitored after 3 h by about 70% and this procedure was then used as a standard ischemic preconditioning in
subsequent studies. In control tests without ischemic preconditioning, 3 days after I/R, chronic gastric ulcers appeared with an area of about 53 mm$^2$. In rats with the ischemic preconditioning the chronic ulcer area was only about 26 mm$^2$ and this reduction as compared to controls was statistically significant (Fig. 2).

The gastric blood flow in the intact stomach averaged 53 ± 6 ml/min/100 g (taken as 100%) and this was significantly reduced (by about 27%) at the end of 3 h of reperfusion that followed 30 min of ischemia. Short ischemic episodes preceding the 30 min I/R resulted in a significant increase of the gastric blood flow to about 81% of the control value (Fig. 1). After 3 days following the I/R, when chronic ulcers occurred, the gastric blood flow returned to about 90% of the value recorded in the intact mucosa and this was not significantly different from that obtained in the gastric mucosa of rats subjected to short ischemic preconditioning before the I/R.

The area of I/R induced acute erosions recorded after 3 h and chronic gastric ulcers found after 3 days after this procedure in rats with inactivated
sensory nerves was significantly higher than that recorded in rats with intact sensory nerves (Figs 1 and 2). The area of both the early acute gastric lesions and chronic gastric ulcerations provoked by I/R in rats with intact sensory nerves were significantly lower than those in animals with inactivated sensory nerves. In tests with ischemic preconditioning, these areas were significantly reduced but this reduction was less pronounced in rats with inactivated sensory nerves than in those with intact nerves. The gastric blood flow following ischemic preconditioning was significantly smaller in rats with inactivated nerves as compared to those with intact sensory nerves.

**CGRP content in gastric mucosa in rats with gastric lesions induced by regular ischemia/reperfusion with or without pretreatment with standard preconditioning**

The CGRP immunoreactivity in the intact gastric mucosa averaged about 34 ± 6 ng/g. Following capsaicin deactivation the content of CGRP was reduced.
by about 70%. Following I/R in rats with intact sensory nerves, there was a significant rise in CGRP content to about 52 ± 5 ng/g after 3 h and to 49 ± 4 ng/g after 3 days upon the submission of rats to I/R but in rats with deactivated sensory nerves the CGRP content after 3 h and 3 days after I/R were significantly decreased below the control values observed in rats with intact sensory nerves. Ischemic preconditioning caused further increase in CGRP content in gastric mucosa both after 3 h and 3 days upon the I/R over that recorded in sham-operated animals without ischemic preconditioning (Table 1).

**DISCUSSION**

This study demonstrates that preconditioning of the gastric mucosa with short episodes of ischemia of the stomach exerts protective action against acute gastric lesions and chronic ulcers caused by longer and more severe exposure to regular I/R and these protective effects are accompanied by an increase in gastric blood flow and are attenuated by the inactivation of sensory nerves.

As mentioned in the introduction, the first evidence of preconditioning phenomenon was obtained in such organs as heart, lungs, liver, kidneys and

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**Table 1.** Effect of standard ischemic preconditioning (IP) on the mucosal generation of CGRP in gastric mucosa exposed to regular ischemia/reperfusion without or with capsaicin sensory deactivation. Results are mean ± SEM of 8—10 rats. Asterisk indicates a significant change as compared to the value obtained in intact gastric mucosa. Cross indicates a significant change as compared to the value obtained in gastric mucosa exposed to ischemia-reperfusion. Double cross indicates a significant decrease as compared to the respective value obtained in gastric mucosa exposed to ischemia/reperfusion without capsaicin treatment.

<table>
<thead>
<tr>
<th>Type of test</th>
<th>CGRP contents (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>34 ± 6</td>
</tr>
<tr>
<td>Without ischemia/reperfusion:</td>
<td></td>
</tr>
<tr>
<td>IP</td>
<td>41 ± 5*</td>
</tr>
<tr>
<td>Capsaicin</td>
<td>11 ± 2*</td>
</tr>
<tr>
<td>With ischemia/reperfusion:</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>36 ± 4*</td>
</tr>
<tr>
<td>I/R</td>
<td>52 ± 5*</td>
</tr>
<tr>
<td>IP + I/R</td>
<td>68 ± 10*</td>
</tr>
<tr>
<td>Capsaicin + I/R</td>
<td>18 ± 3**</td>
</tr>
<tr>
<td>Capsaicin + IP + I/R</td>
<td>22 ± 2**</td>
</tr>
</tbody>
</table>
We recently demonstrated (7) that it also may occur in the stomach resulting in the limitation of the mucosal damage evoked by severe I/R in this organ. Our study showed that gastric preconditioning represents one of the most powerful protective intervention against the damage induced not only by severe I/R but also by various necrotizing substances including 100% ethanol in the stomach (7).

Based on our present results, it is reasonable to assume that gastric preconditioning involves several mediators, especially sensory nerves and CGRP that seem to play a key role in the mechanism of this protection probably by causing vasodilatation and enhancement of the gastric blood flow. This notion is supported by the fact that gastroprotection and accompanying rise in the gastric blood flow induced by gastric preconditioning were significantly lower in rats with capsaicin inactivated sensory neurons, which are responsible for the release of CGRP (25). Furthermore, the contents of immunoreactive CGRP in the gastric mucosa of rats with deactivated sensory nerves were significantly lower than in vehicle-treated animals and that such sensory deactivation significantly attenuated the gastroprotective activity of gastric preconditioning against the lesions induced by I/R. The involvement of CGRP is supported by the fact that concurrent treatment with exogenous peptide to compensate for the deficiency of this endogenous peptide restored the protective efficacy of gastric preconditioning against the damage by I/R.

Previous studies demonstrated that prostaglandins (PG) applied exogenously or generated endogenously in the gastric mucosa, exhibit high activity in preventing the mucosal damage induced by necrotizing substances including boiling water (28, 29). Adaptive cytoprotection was introduced originally by Robert and his associates (28) to describe the protective activity of endogenous PG generated within gastric mucosa in response to mild topical irritants such as 20% ethanol or 5 mM NaCl against severe mucosal damage induced by strong irritants such as 100% ethanol or 25% NaCl. We demonstrated previously (29) that mild irritants offer the cross-protective response, e.g. 5% NaCl was effective in attenuation of damage induced not only by necrotizing 25% NaCl but also by 100% ethanol, while 20% ethanol prevented the damage caused not only by 100% ethanol but also by 25% NaCl. Recently, we reported (7) that ischemic preconditioning exerted cross-protection against necrotizing substances similar to mild irritants and, again, PG have been implicated, at least in part, in this protection. The relative contribution of PG and other gastroprotectors such as CGRP and the relationship between the action of these substances requires further study.

As mentioned in the introduction, the beneficial effects of preconditioning were first demonstrated in the myocardium (2), but it is now evident that this preconditioning protects against posts ischemic damage of the brain, kidney, skeletal muscle and gastrointestinal organs including stomach, small bowel, liver (1—3, 7, 14, 30, 31). Mechanism of protection induced in heart, brain and
kidneys by ischemic preconditioning has not been fully explained but activation of adenosine A1 receptors and ATP-sensitive potassium channels as well as an inhibition of neutrophil activation and emigration in the intestine were implicated in this phenomenon (4). In the stomach, the preconditioning also appears to enhance gastric mucosal resistance against the damage induced by subsequent exposure to prolonged and severe I/R and other mucosal irritants and ulcerogens.

In this study we attempted to determine the role of sensory nerves and their major hormonal mediator, CGRP, in this protection in the stomach. Numerous studies have documented that PG derived from the activity of the cyclooxygenase isoforms, especially cyclooxygenase-1, play an important role in mechanism of gastroprotection (28, 29) and ulcer healing (24). Recently, PG-derived from cyclooxygenase-2 have been also implicated in the protective and ulcer healing activities of growth factors by the demonstration that cyclooxygenase-2 is upregulated in the edge of gastric ulcer and that this is significantly enhanced by the treatment with growth factors (32). Moreover, endogenous PG derived from cyclooxygenase-1 and cyclooxygenase-2 are involved in the mechanism of mucosal recovery from I/R-induced acute gastric erosions that subsequently progressed into deeper ulcerations and that healing of these ulcers is associated with an overexpression of cyclooxygenase-2 mRNA (23). The involvement of PG in the mechanism of preconditioning has not been fully elucidated but it was suggested that in the heart certain PG such as prostacyclin, that is released from ischemic myocardium, may limit the extent of heart infarct and attenuate ventricular arrhythmia and that inhibition of cyclooxygenase prevented the protective effect of ischemic preconditioning in dog myocardium (1, 2). Our results with the preconditioning in the stomach are in keeping with these findings by showing directly that cyclooxygenase-2 is overexpressed in the preconditioned gastric mucosa, at the levels of both, mRNA and protein, while cyclooxygenase-1 mRNA remains unchanged (7).

Previous studies revealed that NO released from vascular endothelium, sensory afferent nerves or gastric epithelium is essential for the gastroprotection and ulcer healing (12, 27, 33—38). We documented previously that administration of NO-synthase inhibitors abolished the gastroprotective activity of capsaicin in the stomach and delayed healing of chronic gastric ulcers (12). Our recent study with NOS inhibitors also suggest that NO could participate as a candidate mediator in gastric preconditioning (7).

We found that the gastroprotection afforded by preconditioning in the stomach, similar to that in the liver induced by hepatic ischemic preconditioning (5, 6), is accompanied by the rise in the blood flow probably due to enhanced production of NO in the gastric mucosa (7). Both these effects occurring after preconditioning were attenuated in rats with suppressed NO synthase activity by L-NNA (7). Similar reversal of protective effects of gastric preconditioning against I/R was observed in rats with capsaicin-induced
deactivation of sensory nerves suggesting that sensory nerves cooperate with NO in the beneficial effects of ischemic preconditioning against severe lesions caused by prolonged I/R. The important role of NO is further supported by the finding that addition to L-NNA of L-arginine, the substrate for NOS activity, but not D-arginine, restored the gastroprotection against I/R, luminal release of NO and the hyperemia evoked by gastric preconditioning (7). Moreover, co-administration of CGRP, together with gastric preconditioning in rats with functionally ablated sensory nerves restored the gastroprotective and hyperemic effects of this preconditioning with the extent comparable to that achieved with preconditioning in rats without capsaicin denervation (40). This suggests that the release of neuropeptides such as CGRP from sensory afferents may mediate the gastroprotection against the ischemia/reperfusion damage induced by preconditioning. This is in keeping with original observation of Ferdinandy et al. (39) suggesting that capsaicin-sensitive afferent nerves are involved in the cardiac preconditioning and confirms that significant portion of NO in the gastric lumen observed in our previous study (7), might also be of neural origin.

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Received: October 3, 2001
Accepted: October 18, 2001

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