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IMPORTANCE OF BRAIN-GUT AXIS IN THE GASTROPROTECTION INDUCED BY GASTRIC AND REMOTE PRECONDITIONING

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Limitation of the damage to the organs such as heart, liver, intestine, stomach and brain by an earlier brief complete occlusion of their arteries is defined as ischemic preconditioning (IP). No study so for has been undertaken to check whether brain-gut axis is involved in the gastroprotection exhibited by gastric IP or in that induced by repeated brief episodes of ischemia of remote organs such as heart and liver. This study was designed to determine the possible involvement of vagal and sensory afferent nerves, in the mechanism of gastric and remote organ IP on the gastric mucosa in rats exposed to prolonged ischemia-reperfusion with or without functional ablation of sensory nerves by capsaicin or in those with removed vagal innervation by vagotomy. This gastric IP was induced by short ischemia episodes (occlusion of celiac artery 1-5 times for 5 min) applied 30 min before subsequent ischemia followed by 3 h of reperfusion (I/R) and compared with remote IP induced by occlusion of left descending coronary artery or hepatic artery plus portal vein. The area of gastric lesions was determined by planimetry, gastric blood flow (GBF) was measured by H_2-gas clearance method and mucosal biopsy samples were taken for the assessment of calcitonin gene-related peptide (CGRP) by RIA. Exposure of gastric mucosa to standard 3 h of I/R produced numerous gastric lesions and significant fall in the GBF and mucosal CGRP content. Two 5 min short ischemic episodes by occlusion of coronary or hepatic arteries, significantly reduced gastric damage induced by I/R with the extent similar to that exhibited by two short (5 min) episodes of gastric ischemia. These protective effects of gastric and remote IPs were accompanied by a restoration of the fall in the CGRP content caused by I/R alone. Protection and hyperemia induced by gastric IP were significantly attenuated in capsaicin-denervated or vagotomized animals and completely removed in those exposed to the combination of vagotomy and capsaicin-denervation. The IP-induced protection and hyperemia were restored by the administration of exogenous CGRP to gastric IP in capsaicin-treated animals. Gastroprotective and hyperemic actions of remote IP were markedly diminished in capsaicin-denervated rats and in those subjected to vagotomy. We conclude that brief ischemia in remote organs such as

heart and liver protects gastric mucosa against gastric injury induced by I/R as effectively as gastric IP *via* mechanism involving both vagal and sensory nerves releasing vasodilatatory mediators such as CGRP.

INTRODUCTION

Ischemia preconditioning (IP) refers to a phenomenon in which a tissue is rendered resistant to the deleterious effect of prolonged severe ischemia by previous exposures to brief moderate vascular occlusions (1). This protective effects of IP were first described in the heart by Murry and coworkers in 1986 (2). Since that time, IP has been shown to reduce the extent of myocardial infarct size as well as the damage of skeletal muscle, brain necrosis or hepatic damage induced by subsequent exposure to severe ischemia in a variety of species (3 - 5).

Recently, we have shown that few short ischemic episodes of gastric mucosa protect this mucosa from the damage induced by prolonged ischemia-reperfusion (I/R) (6, 7). The mechanism of this gastroprotection involves endogenous prostaglandins (PG) derived from COX-1 and COX-2, nitric oxide (NO) due to overexpression of iNOS and adenosine acting on A_1 receptors (6, 7). Our studies (6, 7) provided additional evidence that IP can also exists in the gut and supported original demonstration that IP prevented the mesenteric microvascular barrier dysfunction and activation of excessive amount of NO in the intestine (8 -10). Davenpeck *et al.* (8) reported that IP of rat mesenteric venules led to enhanced bioavailability of nitric oxide (NO) and abolished oxidant metabolites production resulting in the decrease in the leukocyte adhesion and their emigration through mesentery.

The question remains whether similar protective effect of IP could be achieved by remote ischemia of non-gastric organs such as heart or liver resulting in gastroprotection against gastric lesions induced by subjecting of gastric mucosa to longer I/R and if so which mechanism is involved in this response to preconditioning at the distance. Previous studies indicated that preconditioning can induce ischemic tolerance in a variety of organ systems including brain, heart, liver, small intestine, skeletal muscle, kidney and lung (11 - 15). Moreover, it was demonstrated that brief ischemia of remote organs such as liver, skeletal muscle and kidneys protects myocardium as effectively as myocardial preconditioning (11 - 13, 16) but the involvement of brain-gut axis in the gastroprotective action of IP have been little studied.

This study was designed to determine the role of sensory and vagal innervations in the mechanism of gastric preconditioning and in that induced by preconditioning at the distance by short ischemia of coronary and hepatic arteries

in rats with intact vagal and sensory nerves and in those with capsaicin-denervation or vagotomy. An attempt was made to measure of a gastric blood flow (GBF) and CGRP content in gastric mucosa of rats subjected to gastric and remote preconditioning with or without prolonged I/R.

METARIAL 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. All procedures performed in that study were accepted by the Animal Care Local Ethical Committee at the Jagiellonian University Medical College.

Production of gastric lesions induced by ischemia-reperfusion

Three major series (A, B and C) of rats were used. In rats of series A, I/R erosions were produced in 80 rats by the method originally proposed by Wada *et al.* (17). 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 1-5 times for 5 min - IP) was applied 30 min before subsequent exposure to longer 30 min of ischemia (also induced by clamping of celiac artery) and followed by 3 h of reperfusion (I/R). The respective control group included the sham operated control animals, whose the celiac artery was only slightly manipulated but not occluded. In order to compare the effect of preconditioning of remote organs with that produced in the stomach on the lesions induced by I/R, we selected the standard gastric preconditioning (2 x 5 min occlusion of celiac artery) against the gastric erosions caused by I/R. The time of preconditioning was selected on the basis of our previous studies (6, 7, 18), showing that two 5 min ischemic episodes reduced the lesions induced by I/R by about 80%.

Induction of preconditioning of heart and liver

In sereis B, the preconditioning of the heart was induced in phentobarbital-anesthetized open-chest rats by two short (5 min) ischemic episodes of left anterior descending coronary artery (LAD) according the method described by Brinbaum *et al.* (16) in rabbit and adopted by us in rat experiments. Repeated injections of phentobarbital were given during the experiments as required to maintain a deep level of anesthesia. The chest of the rat was opened through the left fourth intercostal space. The pericardium was then incised and the heart exposed. Near the base of the heart, a large anterolateral branch of the left circumflex artery or the artery itself was encircled with a 4-0 silk suture. The ends of the suture were threaded through a piece of tubing, forming a snare that could be tightened to occlude artery. After preconditioning consisting of 2x5 min of LAD occlusions, the laparatomy was performed and the stomach was exposed. Celiac artery was occluded for 30 min (ischemia) followed by reperfusion lasting 3 h as described in detail above.

In rats of seriec C, hepatic preconditioning was induced by occluding the common hepatic artery and portal vein with a metal vascular clips twice for 5 min and reperfusion was allowed for 10 min after each of 5 min ischemic episode (18). Then, rats were subjected to 30 min of gastric ischemia followed by 3 h of reperfusion according the procedure described above.

Implication of sensory afferent and vagal nerves in gastric IP induced by short ischemia

In tests with sensory afferent nerves and neuropeptides in gastroprotection by gastric and remote preconditioning, rats with capsaicin-induced deactivation of these nerves with or without addition of exogenosus CGRP or those pretreated with calcitonin gene related peptide (CGRP) receptor antagonist, CGRP$_{8-37}$, respectively (19, 20) were used. 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 experiment as described previously (21). 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 0.1mg/ml solution of capsaicin was instilled into the eye of each rat and the protective movements were counted as described previously (19). 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. IP of remote organs was compared with that of the stomach in rats with intact or capsaicin deactivated nerves and this was followed by 3 h of I/R according to the procedure described above. In another
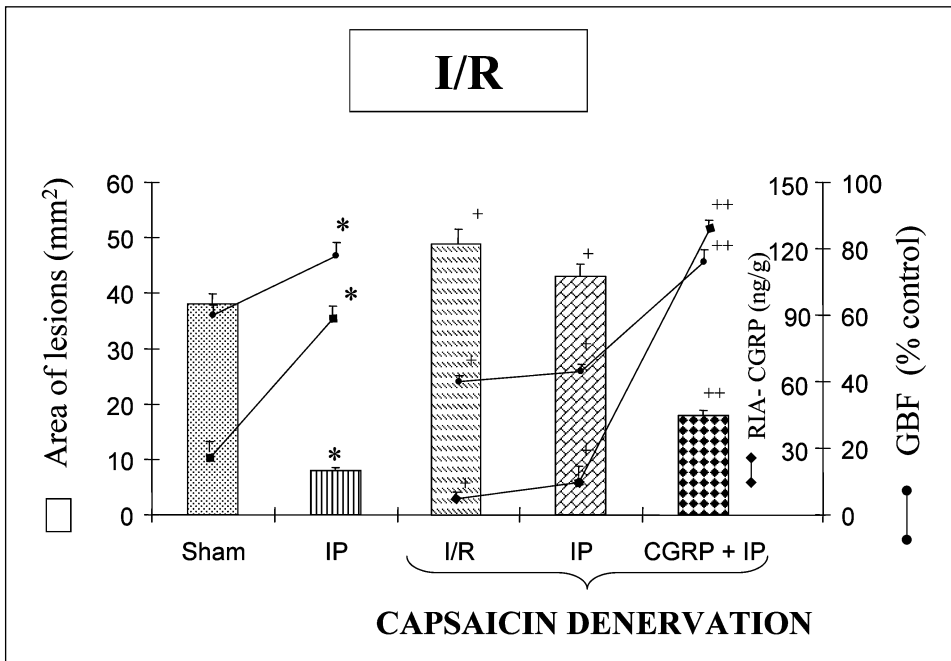

Fig. 1. Effect of gastric ischemic preconditioning (IP) in rats with intact or capsaicin-deactivated sensory nerves with or without addition of calcitonin gene-related peptide (CGRP, 10 μg/kg i.p.) on the area of gastric lesions (columns) and accompanying changes in the gastric blood flow (GBF) and mucosal CGRP content (lines) caused by the exposure to 30 min of ischemia followed by 3 h of reperfusion (I/R). Results are mean ± SEM of 6-8 rats. Asterisk indicates a significant change as compared with the value obtained in sham-control gastric mucosa. Cross indicates a significant change as compared with the value obtained in rats without capsaicin denervation. Double cross indicates a significant change as compared with the value obtained in capsaicin-denervated rats.
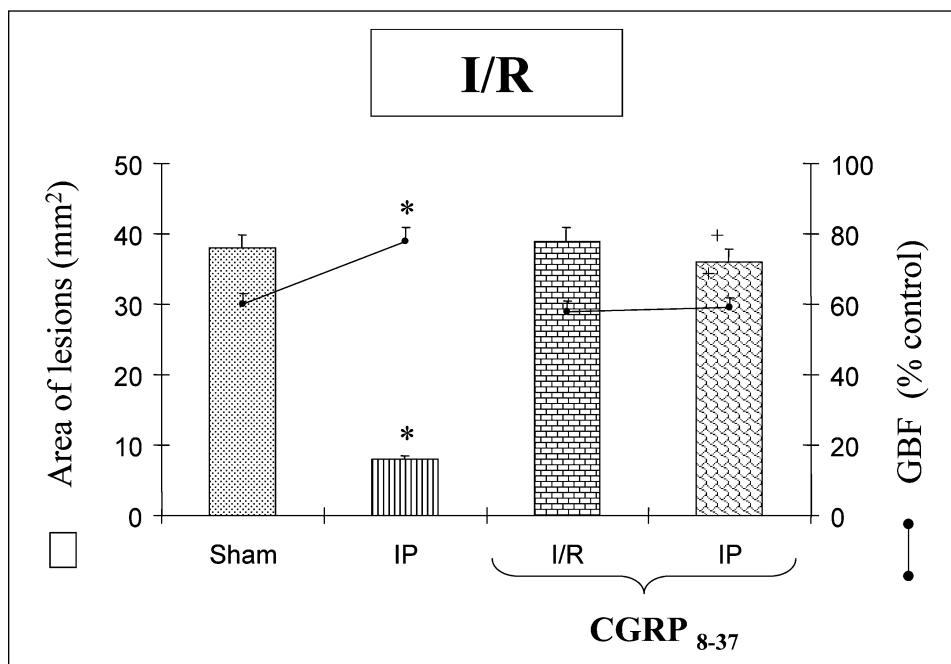
Fig. 2. Effect of gastric ischemic preconditioning (IP) with or without the pretreatment with CGRP receptor antagonist, CGRP$_{8-37}$ (100 µg/kg i.p.) on the area of gastric lesions and accompanying changes in the gastric blood flow (GBF) induced by the exposure to ischemia-reperfusion (I/R). Results are mean ± SEM of 6-8 rats. Asterisk indicates a significant change as compared with the value obtained in sham-control gastric mucosa. Cross indicates a significant change as compared with the value obtained in rats without CGRP$_{8-37}$ pretreatment.

group of capsaicin denervated rats, CGRP (10 µg/kg s.c.) was applied s.c. 30 min before short ischemia of heart, liver and stomach followed by exposure of gastric mucosa to I/R, in order to check, whether deficiency of endogenous 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 (19).

The CGRP receptor antagonist CGRP$_{8-37}$ was applied 30 min before standard IP or vehicle followed 30 min later by I/R. The experimental protocol included the following study groups, each consisting of 6-8 animals; 1) vehicle (saline) followed 30 min later by gastric IP in rats with intact afferent nerves; 2) gastric IP followed 30 min later by I/R in rats with intact sensory nerves; 3) vehicle (saline) followed 30 min later by I/R in rats with capsaicin-deactivated afferent nerves or those pretreated with CGRP$_{8-37}$ (100 µg/kg i.p.); 4) gastric IP followed 30 min later by I/R in rats with capsaicin deactivated afferent nerves or those pretreated with CGRP$_{8-37}$ (100 µg/kg i.p.).

The involvement of vagal nerves in IP-induced gastroprotection was studied in rats with or without subdiaphragmatic vagotomy after cutting off vagal nerves as described previously (22). About 30 min before the experiment, rats were anesthetized with ether and abdomen was opened by small incision. Both branches of vagal nerves were identified at the level of diaphragm and finally cut off. The control rats were treated similarly except the vagi were only uncovered but left intact. Vagotomized and sham-operated rats were subjected to gastric IP followed 30 min later by

prolonged I/R. Then rats were sacrificed and area of gastric lesions and GBF were measured in similar manner as mentioned above.

Measurement of gastric blood flow (GBF) and determination of CGRP by RIA

At the termination of each experiment, the GBF was measured by H_2-gas clearance technique. Rats were lightly anesthetized with ether, the abdomen was opened and the stomach was exposed. The GBF was measured in the oxyntic gland area of the stomach by means of local H_2-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/100g-min) of these measurements were calculated and expressed as percent changes from those recorded in control animals treated with vehicle.
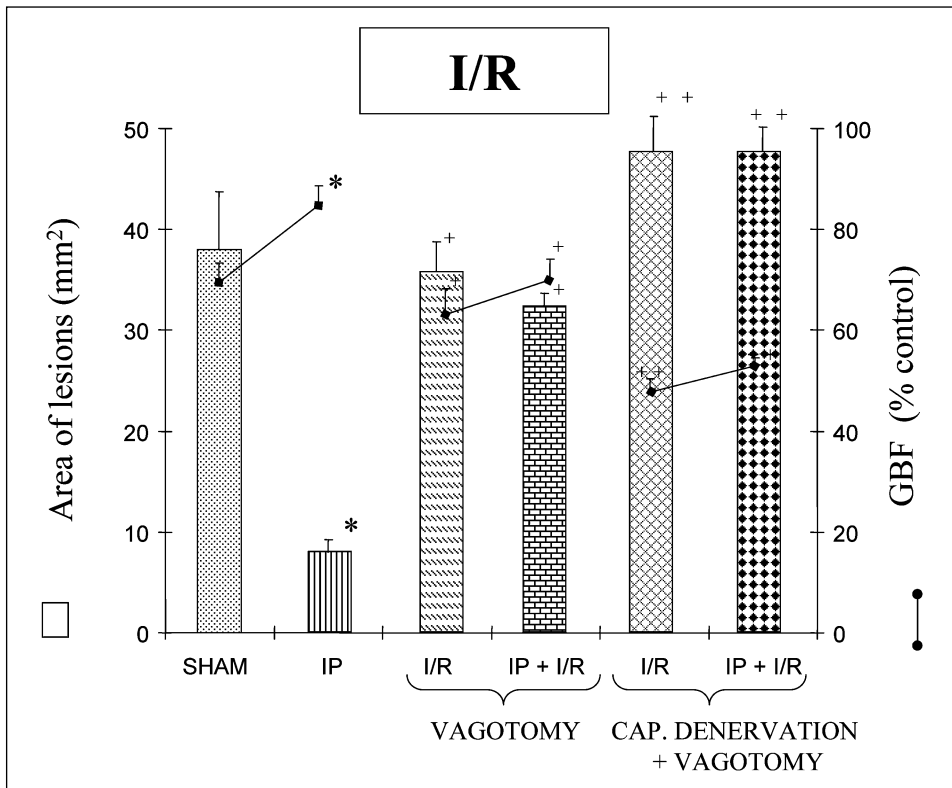

Fig. 3. The area of ischemia-reperfusion (I/R)-induced gastric lesions and the alterations in the gastric blood flow (GBF) in sham (control) rats or those pretreated with gastric ischemic preconditioning (IP) with or withot vagotomy or the combination of vagotomy and capsaicin denervation. Subdiaphragmatic vagotomy was performed about 30 min prior to the gastric IP while deactivation of sensory nerves with large dose of capsaicin (125 mg/kg s.c.) was done 14 days prior to start of the experiment with gastric IP. Mean ± SEM of 6-8 rats. Asterisk indicates a significant change compared to the value in sham-control rats. Cross indicates a significant change compared to the value obtained in rats without vagotomy. Double cross indicates a significant change as compared with the value obtained in rats with or without vagotomy alone.

Gastric mucosal biopsy samples (about 100 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, USA in accordance with the manufacturer's recommendation. 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.

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 capsaicin denervation and administration of $CGRP_{8-37}$ on gastric lesions induced by standard I/R and GBF in rats with or without pretreatment with gastric IP

As shown in *Fig. 1*, gastric IP consisiting of 2 times 5 min short ischemic episodes profoundly attenuated the I/R-induced gastric lesions and significantly raised the GBF as compared to the respective values in gastric mucosa exposed to I/R alone without IP. The CGRP immunoreactivity in the intact mucosa averaged 42±4 ng/g and this was significantly decreased in gastric mucosa of rats exposed to I/R. Pretreatment with IP significantly increased the mucosal CGRP immunoreactivity as compared to that in animals exposed to I/R alone. Capsaicin denervation increased significantly the I/R-induced gastric lesions and this effect was accompanied by a marked fall in the GBF and mucosal CGRP content. The protective and hyperemic effects of IP were significantly attenuated in rats with sensory nerve deactivation attained with pretreatment with capsaicin and this effect was accompanied by the significant fall in the CGRP content. Addition of exogenous CGRP to capsaicin denervation, restored in part, the protective effect and accompanying increase in the GBF and CGRP content evoked by short ischemia of stomach against lesions caused by I/R (*Fig. 1*).

The pretreatment with $CGRP_{8-37}$, which by itself tended to increase, though not significantly, the lesions induced by I/R, almost completely abolished the decrease of the gastric lesions and accompanying increase in the GBF produced by IP (*Fig. 2*).

Effect of vagotomy with or without capsaicin denervation on the gastroprotection and changes in the GBF induced by IP

As shown in *Fig. 3*, gastric IP consisting of 2 times 5 min short ischemic episodes attenuated the I/R-induced gastric lesions and raised the GBF, similarly

as presented in *Figs 1* and *2*. Vagotomy failed to influence the area of I/R-induced gastric lesions, but significantly reduced the GBF, reversed the IP-induced gastroprotection and the accompanying increase in the GBF. When vagotomy was combined with capsaicin-denervation, the protective and hyperemic effects of gastric IP were completely abolished (*Fig. 3*).

Effect of remote IP on the gastric lesions and changes in the GBF induced by gastric I/R in rats with or without capsaicin-denervation or vagotomy.

Fig. 4 shows the effect of two short (5 min) gastric, hepatic or cardiac ischemic episodes on the area of gastric lesions and changes in the GBF induced by prolonged gastric I/R. Two 5 min gastric preconditioning episodes reduced the area of I/R erosions by about 87%. The coronary artery and hepatic artery occlusion twice, resulted in a significant reduction in lesion area caused by I/R with the magnitude similar to that achieved with gastric IP. Gastric IP increased significantly
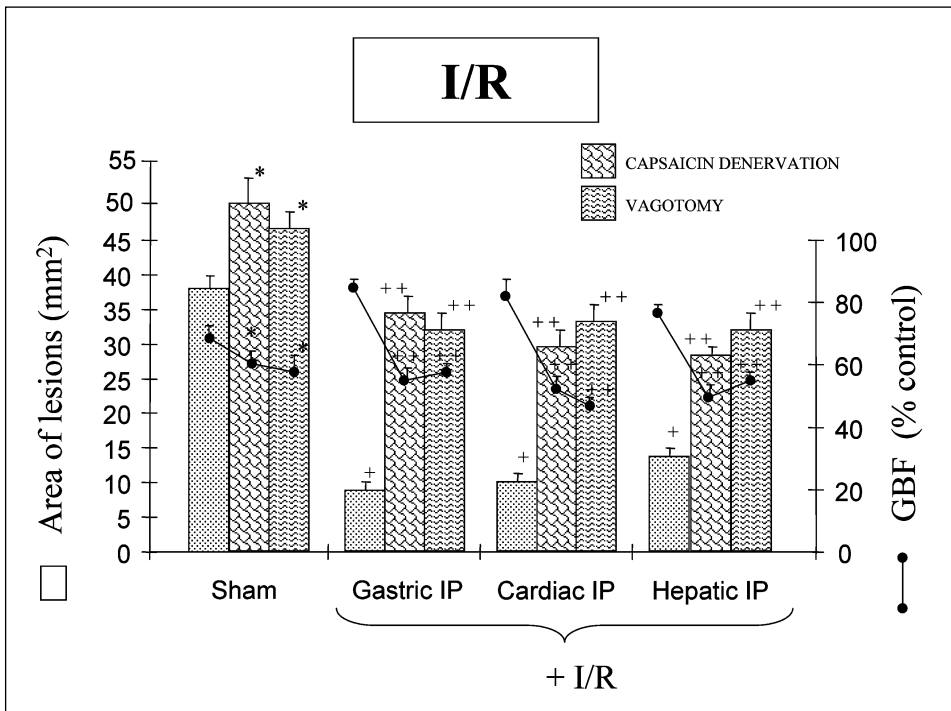

Fig. 4. The area of ischemia-reperfusion (I/R)-induced gastric lesions and the alterations in the gastric blood flow (GBF) in sham (control) rats or those pretreated with gastric, cardiac and hepatic ischemic preconditioning (IP) with or without vagotomy or capsaicin denervation. Mean ± SEM of 6-8 rats. Asterisk indicates a significant change compared to the value in sham-control rats without vagotomy or capsaicin denervation. Cross indicates a significant change compared to the value obtained in sham-control without IP. Double cross indicates a significant change as compared with the value obtained in rats without vagotomy or capsaicin denervation.

the GBF by about 30% as compared to that recorded in sham-operated controls exposed to I/R and these values was not significantly different from that obtained in gastric mucosa of rats subjected to short ischemia of heart or liver.

Both, capsaicin denervation and vagotomy significantly increased the I/R-induced gastric lesions and these effects were accompanied by a significant fall in the GBF. The gastroprotective and hyperemic activities of gastric, cardiac and hepatic IPs, which caused usual significant decrease in area of I/R-induced gastric lesions and the rise in the GBF in rats with intact sensory or vagal nerves, were significantly attenuated in vagotomized animals and those with sensory nerve deactivation attained with pretreatment with capsaicin (*Fig. 4*).

<div align="center">DISCUSSION</div>

This study confirms our original observations that preconditioning of the gastric mucosa with short episodes of ischemia of the stomach exerts protective action against acute gastric lesions caused by severe I/R and that these protective effects of gastric IP are accompanied by an increase in gastric blood flow (6, 7). The major finding of our present study is the observation that gastric IP-induced protection and accompanying increase in the GBF are markedly attenuated in rats with vagotomy and capsaicin-denervation suggesting that the mechanism of this gastroprotection evoked by gastric IP involves the activation of brain-gut axis. Another important finding of this study is that brief ischemia of the remote organs such as heart or the liver exerted the protective influence on gastric mucosa injured by prolonged I/R with the extent similar to that observed with gastric IP. To our best knowledge, this is the first demonstration that phenomenon of gastroprotection against damage induced by severe I/R by means of preconditioning at the distance induced by short ischemic episodes to non-gastric tissue.

As mentioned in the "Introduction", the first evidence of preconditioning phenomenon has been obtained in various organs including heart, lungs, liver and intestine (1 - 4, 8, 10, 18). We have recently demonstrated that this phenomenon may occur also in the stomach and it could be considered as a powerful intervention in the stomach resulting in limitation of the extent of mucosal damage evoked by the I/R (6, 7).

We found that remote preconditioning of heart and liver induced in these organs by short episodes of LAD occlusion or combined occlusion of hepatic artery and portal vein, respectively, prevented the gastric mucosal lesions caused by severe gastric I/R mimicking the gastroprotective effect of short ischemia episodes of the gastric artery in the stomach. Moreover, we documented that this gastroprotective effect of remote IP involves the comparable increase in the gastric blood flow as in case of gastric IP.

Based on these results, it is reasonable to assume that gastric and remote IPs that resulted in gastroprotection, involve neural mediators including vagal nerves and neuropeptides released from sensory nerves such as CGRP, that is known to induce vasodilatation and enhancement of the GBF (6, 24, 26). This notion is supported by the fact that gastroprotection and accompanying rise in the GBF induced either by gastric, cardiac and hepatic IPs were significantly attenuated in rats with functional ablation of sensory nerves by capsaicin as well as in those with vagotomy. This suggests that both, sensory afferent nerves that were shown to release NO and various vasodilatatory neuropeptides such as CGRP (21, 24, 27- 30) and vagal efferent nerves play an important role in the mechanism of this gastroprotection. In accordance with this suggestion, our present evidence revealed that the combination of vagotomy and capsaicin denervation, which aggravated the I/R-induced gastric lesions, resulted in a complete elimination of the IP-induced gastroprotection and accompanying hyperemia. Moreover, the concurrent treatment with exogenous CGRP to replace the neuropeptide lost by deactivation with capsaicin-sensitive afferent nerves, counteracted the deleterious effects of capsaicin-induced denervation in preconditioned gastric mucosa exposed subsequently to I/R.

Two types of protection afforded by preconditioning stimuli, namely, the acute and delayed preconditioning were recently distinguished (27 - 30, 33), but little information is available as to whether preconditioning of remote organs could influence the gastric lesions induced by I/R. It has been demonstrated that myocardial protection can be conferred by an erlier brief ischemia of a remote myocardial region (preconditioning at the distance) or even by brief ischemia of a remote organ, such as the kidney or the mesentery (11). Remote preconditioning induced by occlusion of renal artery afforded protection against myocardium damage induced I/R and facilitated better recovery of ATP during reperfusion as compared to that in non-preconditioned myocardium (11). Tang *et al.* (31) have demonstrated that brief ischemia of the small intestine induced both early and delayed protection against I/R myocardial injury and that this protective effects depend upon the activation of capsaicin-sensitive nerves. Finally, remote ischemia of a skeletal muscle induced by muscle stimulation combined with the restriction of the blood flow resulted in a considerable reduction of myocardial infarct size (16). All these studies supported the notion that brief ischemia in the remote organs protects the myocardium against infarction as effectively as myocardial preconditioning (11, 16, 29, 33).

According to our knowledge no attempts were made to examine the effect of preconditioning of remote organs such as heart or the liver could attenuate the mucosal damage induced in the stomach by I/R. Therefore, we propose that mechanism of remote preconditioning in the stomach is attributed to the activation of sensory and products released from these nerves as potential mediators of this protection and require intact vagal innervation. This is supported by our finding that the IP-induced protection and hyperemia were greatly reduced

in rats with capsaicin-induced deactivation of sensory nerves suggesting that in addition to NO, also sensory and vagal nerves cooperate in the beneficial effects of short ischemia against severe lesions caused by prolonged I/R. In agreement with this notion, co-administration of CGRP, together with gastric IP in rats with functionally ablated sensory nerves restored the gastroprotection and hyperemia with an effect comparable to that achieved with gastric IP in rats without capsaicin denervation. This suggests that the release of neuropeptides such as CGRP from sensory afferents mediates the gastroprotection against the I/R damage induced by remote preconditioning. This is in keeping with recent observations in heart indicating that capsaicin-sensitive afferent nerves are involved in the cardiac preconitioning (32, 34) and that cardioprotection achieved by remote intestinal preconditioning could mediated by endogenous CGRP (35) also suggesting that significant portion of NO in the heart and gastric lumen as observed in our previous studies (6, 7, 18), might be of neural origin.

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