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European Journal of Pharmacology 499 (2004) 201–213

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Ischemic preconditioning of remote organs attenuates gastric ischemia–reperfusion injury through involvement of prostaglandins and sensory nerves

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Received 8 July 2004; accepted 13 July 2004
Available online 1 September 2004

Abstract

Limitation of the stomach damage by its earlier brief ischemia and reperfusion before prolonged ischemia is defined as gastric ischemic preconditioning but whether such brief ischemia of remote organs like heart or liver can also attenuate the gastric damage caused by longer and severe ischemia–reperfusion remains unknown. The cardiac, hepatic and gastric preconditioning were induced by brief ischemia (occlusion of coronary, hepatic and celiac arteries twice for 5 min) applied 30 min before 3 h of ischemia/reperfusion. Standard 3 h ischemia–reperfusion of the stomach produced numerous gastric lesions, decreased gastric blood flow and mucosal prostaglandin E_2 generation and increased expression and plasma release of interleukin-1β and tumor necrosis factor-alpha (TNF-α). These effects were significantly attenuated by brief cardiac, hepatic and gastric preconditioning which upregulated cyclooxygenase-2 mRNA but not cyclooxygenase-1 mRNA. The protective effects of brief gastric, cardiac and hepatic preconditioning were attenuated by selective cyclooxygenase-1 and cyclooxygenase-2 inhibitors and capsaicin denervation. We conclude that brief ischemia of remote preconditioning such as heart or liver protects gastric mucosa against severe ischemia–reperfusion-induced gastric lesions as effectively as local preconditioning of the stomach itself via the mechanism involving prostaglandin derived from cyclooxygenase-1 and cyclooxygenase-2 and the activation of sensory nerves releasing calcitonin gene-related peptide (CGRP) combined with the suppression of interleukin-1β and TNF-α expression and release.

Keywords: Gastric preconditioning; Ischemia–reperfusion; Gastric blood flow; Remote preconditioning; Prostaglandin; Sensory nerve

1. Introduction

Ischemic preconditioning refers to a phenomenon in which a tissue is rendered resistant to the deleterious effect of prolonged and severe ischemia by previous exposures to brief and moderate vascular occlusions (Parratt, 1994). These protective effects of ischemic preconditioning were first described in the heart by Murry et al. (1986). Since that time, ischemia preconditioning 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 (Mounsey et al., 1992; Ishida et al., 1997; Nilsson et al., 2000; Peralta et al., 2000). Recently, we have shown that the exposed of stomach to few brief ischemic episodes protects the gastric mucosa from the damage induced by severe, prolonged ischemia–reperfusion (Pajdo et al., 2001). The mechanism of this gastroprotection was found to involve endogenous prostaglandins derived from activation of cyclooxygenase-1 and cyclooxygenase-2, nitric oxide (NO) due to overexpression of iNOS and adenosine acting

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doi:10.1016/j.ejphar.2004.07.072

on A1 receptors (Pajdo et al., 2001; Konturek et al., 2001). Other studies documented that such ischemic preconditioning can also exists in the gut as evidenced by the demonstration that ischemic preconditioning prevented the mesenteric microvascular barrier dysfunction and activation of excessive amount of NO in the intestine (Davenpeck et al., 1994; Hotter et al., 1996; Russell et al., 1996; Tsuruma et al., 1996).

The question remains whether brief ischemic preconditioning of non-gastric organs such as heart or liver could also result in gastroprotection against gastric lesions induced by severe ischemia–reperfusion and if this cross protection can be induced by preconditioning of remote organs. Previous studies indicated that ischemic preconditioning raises directly the tolerance to damage of various organ systems including brain, heart, liver, small intestine, skeletal muscle, kidney, lung and pancreas. Moreover, it was demonstrated that brief ischemia of remote organs such as liver and intestine may protect myocardium as effectively as myocardial preconditioning itself (Gho et al., 1996).

This study was designed to determine whether preconditioning at the distance by short ischemia of heart or liver can protect the gastric mucosa from lesions induced by severe ischemia-reperfusion and if so to elucidate the contribution of endogenous prostaglandins by using rats pretreated with or without a non-selective cyclooxygenase inhibitor (indomethacin) or selective cyclooxygenase-1 inhibitor 5-(4-chlorophenyl)-1-(4-metoxyphenyl)-3-(trifluoromethyl)-1*H*-pyrazole (SC-560) or highly selective cyclooxygenase-2 inhibitor (rofecoxib). In addition, the involvement of sensory nerves to this gastroprotective effect of remote preconditioning was determined in rats subjected to capsaicin-induced functional ablation of these nerves. An attempt was made to assess the mucosal gene expression of cyclooxygenase-1 and cyclooxygenase-2 and proinflammatory cytokines such as interleukin-1β and TNF-α in gastric mucosa subjected to direct gastric and remote organ preconditioning with or without prolonged gastric ischemia–reperfusion.

2. Materials and methods

Male Wistar rats weighing 220–250 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 accomplished according to Helsinki Declaration and accepted by the Animal Care Local Ethical Committee at the Jagiellonian University Medical College.

2.1. Production of gastric lesions induced by ischemia–reperfusion

Three major series (A, B and C) of rats were used. In rats of series A, standard ischemia–reperfusion erosions were produced in 120 rats by the method originally proposed by Wada et al. (1996). Briefly, under pentobarbital anesthesia (50 mg/kg i.p.), the abdomen was opened, the celiac artery identified and clamped with a small clamp for 30 min followed by removal of this clamp to obtain reperfusion for 3 h. In addition, brief ischemia (occlusion of celiac artery twice for 5 min—ischemia preconditioning with two times 10 min reperfusion after each 5 min ischemia episode) was applied 30 min before subsequent exposure to 30 min ischemia (also induced by clamping of celiac artery) and followed by 3 h of reperfusion (ischemia–reperfusion). The respective control group included the sham-operated control animals, whose the celiac artery was only slightly manipulated but not occluded. The time of gastric preconditioning was selected on the basis of our previous study, showing that two 5-min ischemic episodes remarkably (by about 80%) reduced the lesions induced by prolonged (30 min) ischemia followed by 3 h of reperfusion (Pajdo et al., 2001). In order to compare the effect of preconditioning of remote organs with that produced directly in the stomach on the lesions induced by standard ischemia–reperfusion, we selected the gastric preconditioning (2×5 min occlusion of celiac artery) against the gastric erosions caused by standard prolonged ischemia–reperfusion.

2.2. Induction of preconditioning at the distance by ischemia of heart and liver

Preconditioning of the heart was induced in phenobarbital-anesthetized open-chest rats (series B) by two short (5 min) occluding episodes of left anterior descending coronary artery according the method described by Brinbaum et al. (1997) in rabbit and adopted by us in rat experiments. Repeated injections of phenobarbital were given during the experiments as required to maintain a deep level of animal anesthesia. The artificial respiration was assured by the respiratory pump and 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. The sham-operated rats, which also received artificial respiration, were treated identically except that cardiac artery was not occluded. About 30 min following heart preconditioning consisting of 2×5 min of left anterior descending coronary artery 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 details above.

In rats of series C, liver preconditioning was induced by occluding the common hepatic artery and portal vein with a metal vascular clips twice for 5 min with reperfusion for 10 min after each of 5 min ischemic episode (Peralta et al.,

1999; Nilsson et al., 2000). Then, rats were subjected to a standard 30 min gastric ischemia followed by 3 h of reperfusion according the procedure described above.

2.3. Effect of suppression of cyclooxygenase-1 and cyclooxygenase-2 activity on gastric preconditioning

In separate group of rats, the pretreatment with cyclooxygenase inhibitors was employed 30 min prior to cardiac, hepatic and gastric preconditioning followed by prolonged ischemia–reperfusion in order to determine whether suppression by non-selective or selective inhibitors of cyclooxygenase-1 and cyclooxygenase-2 can influence the protective action of distant organ preconditioning.

Several groups of rats, each consisting of six to eight animals, were given 30 min before gastric, hepatic or cardiac preconditioning one of the following treatments: (1) vehicle (saline); (2) SC-560, a potent selective cyclooxygenase-1 inhibitor (Smith et al., 1998); (3) rofecoxib, a highly selective cyclooxygenase-2 inhibitor (Brzozowski et al., 1999); and (4) indomethacin (5 mg/kg i.p.), a non-selective cyclooxygenase inhibitor (Wallace et al., 1998). At the dose used in present study, indomethacin administered intraperitoneally has been shown previously to inhibit gastric prostaglandin E_2 generation capability by about 90% without causing by itself any gross gastric mucosal damage (Konturek et al., 1998). Rofecoxib, a selective cyclooxygenase-2 inhibitor, is well absorbed in the gastrointestinal tract with the peak plasma concentration occurring 2–3 h after oral administration with the bioavailability of about 93% after oral application of the drug in a form of tablette or suspension (Scott and Lamb, 1999). SC-560 is one of the diaryl heterocycle of cyclooxygenase inhibitors which includes celecoxib and rofecoxib. However, unlike these clinically used selective cyclooxygenase-2 inhibitors, SC-560 is a selective inhibitor of cyclooxygenase-1. SC-560 shows 700-fold selectivity for cyclooxygenase-1 and was reported to be orally active in the rat causing at the dose of 10 mg/kg, a complete suppression of tromboxane B_2 in whole blood (Smith et al., 1998). This selective cyclooxygenase-1 inhibitor failed to produce gastrointestinal damage in animals, even when given at a dose of 100 mg/kg (Takeuchi et al., 1994). SC-560 (Cayman Chemical, Ann Arbor, MI, USA). It was first dissolved in absolute ethanol to obtain the stock solution of 50 mg/ml and then diluted to the desired concentration with the isotonic saline. Rofecoxib (Merck Sharp and Dohme, Haar, Germany) was first dissolved in methanol to obtain the stock solution 50 mg/ml and then diluted to the desired concentration with isotonic saline as described previously (Brzozowski et al., 1999). SC-560 and rofecoxib were used in a dose 10 mg/kg i.g. that was shown previously to inhibit the prostaglandin E_2 generation in the inflamed gastrointestinal mucosa (Brzozowski et al., 1999; Tanaka et al., 2002). Control rats received the corresponding vehicle. Our preliminary studies (data not shown) confirmed that none of the cyclooxygenase inhibitors used in this study produced by itself any gastric lesions at the doses tested.

In another group of animals subjected to ischemia preconditioning and then to standard ischemia–reperfusion with or without treatment with cyclooxygenase-1 and cyclooxygenase-2 inhibitors, the prostaglandin tissue deficiency was compensated using 16,16 dimethyl prostaglandin E_2 (Upjohn, Kalamazoo, MI, USA) applied in a dose of 1 μg/kg (i.g.) that was found in our preliminary study to be without any influence on gastric lesions caused by ischemia–reperfusion and accompanying fall in gastric blood flow (data not shown). For this purpose, 16,16 dimethyl prostaglandin E_2 analog was administered together with cyclooxygenase-1 or cyclooxygenase-2 inhibitor starting 30 min prior to standard heart, liver or gastric preconditioning that was followed by 3 h of ischemia–reperfusion.

2.4. Implication of sensory afferent nerves and calcitonin gene-related peptide (CGRP) in gastric preconditioning induced by short ischemia

In tests with sensory afferent nerves and neuropeptides in gastroprotection by direct gastric and remote preconditioning, rats with capsaicin-induced deactivation of these nerves with or without addition of exogenous CGRP (Brzozowski et al., 1999; Ichikawa et al., 2000; Tubaro et al., 2000) were used. For this purpose, the animals were pretreated with capsaicin (Sigma, St. Louis, MO, USA) injected s.c. for 3 consecutive days at doses of 25, 50 and 50 mg/kg about 2 weeks before the experiment as described previously (Takeuchi et al., 1994). 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 (Brzozowski et al., 1996b). 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. Preconditioning of remote organs was compared with that induced directly in the stomach of rats with intact or capsaicin deactivated nerves and this was followed by prolonged ischemia–reperfusion according to the standard procedure described above. In another group of capsaicin denervated rats, CGRP (10 μg/kg s.c.) was applied 30 min before brief ischemia (preconditioning) of heart, liver and stomach followed by exposure of gastric mucosa to standard ischemia–reperfusion, in order to check whether deficiency of endogenous CGRP due to functional ablation of sensory nerves could be compensated by administration of exogenous neuropeptide at a dose that was reported to reverse the effects of capsaicin deactivation on gastric mucosal injury (Brzozowski et al., 1996b).

2.5. Measurement of gastric blood flow and determination of mucosal generation of prostaglandin E_2 and plasma levels of interleukin-1β and TNF-α

At the termination of each experiment, the gastric blood flow was measured by hydrogen (H_2)-gas clearance technique in fully anesthetized rats after abdomen opening and exposure of their stomachs. The gastric blood flow was measured in the oxyntic gland area of the stomach by means of local H_2-gas clearance method using double electrodes of an electrolytic regional blood flow meter (Biomedical Science, Model RBF-2, Osaka Japan) inserted through the serosal into the mucosa. One of these electrodes was used for the local generation in the mucosa of H_2 and another for measurement of tissue H_2. With this method, the H_2 generated locally is carried out by flow of blood, while the polarographic current detector reads out decreasing tissue H_2. The clearance curve of tissue H_2 was used to calculate an absolute blood flow rate (ml/min/100 g) in the oxyntic gland area as described previously (Brzozowski et al., 1999). The measurements were calculated in three areas of the mucosa and the mean absolute values of these measurements were calculated and expressed as percent changes from those recorded in control animals treated with vehicle.

Immediately after gastric blood flow measurement, a venous blood sample was withdrawn from the vena cava into EDTA-containing vials and used for the determination of plasma interleukin-1β and TNF-α and by solid phase sandwich Enzyme-Linked Immunosorbent Assay (Bio-Source International, Camarillo, CA, USA) according to the manufacturer's instructions.

Rats were sacrificed, the stomach was quickly removed and opened along the greater curvature. The gastric mucosa was photographed to subsequently measurement by planimetry (Morphomat, Carl Zeiss, Berlin, Germany) of the area of gastric lesions by two observers according to the method described in detail previously (Brzozowski et al., 1996a; Konturek et al., 1999).

In groups of rats exposed to gastric ischemic preconditioning followed by prolonged standard ischemia–reperfusion without or with pretreatment with cyclooxygenase inhibitors, the mucosal samples were taken by biopsy (about 200 mg) from grossly unchanged gastric mucosa without mucosal lesions immediately after the animals were sacrificed to determine the mucosal generation of prostaglandin E_2 by radioimmunoassay (RIA) as described previously (Konturek et al., 1998). The mucosal samples were placed in preweighed Eppendorf vial and 1 ml of Tris buffer (50 mm, pH 9.5) was added to each vial. The samples were finely minced (during 15 s) with scissors, washed and centrifuged for 10 s, the pellet being resuspended again in 1 ml of Tris. Then, each sample was incubated on a vortex mixer for 1 min and centrifuged for 15 s. The pellet was weighed and the supernatant was transferred to a second Eppendorf vial containing indomethacin (10 mm) and kept at −20 °C until the radioimmunoassay. The capability of the mucosa to generate prostaglandin E_2 was expressed in nanograms of wet tissue weight.

2.6. Expression of cyclooxygenase-1, cyclooxygenase-2, interleukin-1β and TNF-α mRNA transcripts in the gastric mucosa determined by reverse transcriptase-polymerase chain reaction

The mRNAs for cyclooxygenase-1, cyclooxygenase-2, interleukin-1β and TNF-α were determined by reverse transcriptase-polymerase chain reaction in the gastric mucosa of intact rats or those exposed to ischemia–reperfusion with or without remote or gastric ischemic preconditioning. Samples of the gastric oxyntic mucosa (about 200 mg) were scraped off on ice using glass slide and then immediately snap frozen in liquid nitrogen, and stored at −80 °C. Total RNA was isolated from the gastric oxyntic mucosa according to Chomczynski and Sacchi (1987) using a rapid guanidinum isothiocyanate/phenol chloroform single step extraction kit from Stratagene (Heidelberg, Germany).

First-strand cDNA was synthesized from total cellular RNA (5 μg) using 200 U Strata Script TM reverse transcriptase and oligo (dt) primers (Stratagene). The primers for cyclooxygenase-1 and cyclooxygenase-2 were synthesized by Biometra (Gottingen, Germany). The primer sequences were designed according to the published cDNA sequence for the rat β-actin and cyclooxygenases (Nudel et al., 1983; Kennedy et al., 1993; Xie et al., 1991; Feng et al., 1995). The cyclooxygenase-1 primer sequences were as follows: upstream, 5′ -AGC CCC TCA TTC ACC CAT CAT TT; downstream, 3′ -CAG GGA CGC CTG TTC TAC GG. The expected length of this PCR product was 561 bp. The cyclooxygenase-2 primer sequences were as follows: upstream, 5′ -ACA ACA TTC CTT CCT TC; downstream, 3′ -CCT TAT TTC CTT TCA CAC C. The expected length of this PCR product was 201 bp. The interleukin-1β primer sequences were as follows: upstream, 5′ GCT ACC TAT GTC TTG CCC GT; downstream, 3′ GAC CAT TGC TGT TTC CTA GG. The expected length product was 543 bp. The TNF-α primer sequences were as follows: upstream, 5′ TAC TGA ACT TCG GGG TGA TTG GTC C; downstream, 3′ CAG CCT TGT CCC TTG AAG AGA ACC. The expected length product was 295 bp. Concomitantly, amplification of control rat β-actin was performed on the same samples to verify the RNA integrity. DNA amplification was carried out under the following conditions; denaturation at 94 °C for 1 min, annealing at 60 °C for 45 s and extension at 72 °C for 45 s. Each PCR product (8 μl) was electrophoresed on 1.5% agarose gel stained with ethidium bromide, and then visualized under UV light. Location of predicted PCR product was confirmed by using a 100-base pair ladder (Gibco BRL/Life Technologies, Eggenstein, Germany) as standard marker. Comparisons between different treatment groups were made by determining the cyclooxygenase-1 and cyclooxygenase-2, interleu-

kin-1β and TNF-α/β-actin ratio of the immunoreactive area by densitometry.

2.7. Statistical analysis

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

3. Results

3.1. Effect of brief gastric, hepatic and cardiac ischemic episodes on the gastric lesions induced by prolonged (standard) ischemia–reperfusion and the accompanying changes in the gastric blood flow

Fig. 1 shows the effect of two brief (5 min) gastric, hepatic or cardiac ischemic episodes on the area of gastric erosions and changes in the gastric blood flow induced by standard ischemia–reperfusion. Two 5 min of gastric ischemia reduced the area of ischemia–reperfusion gastric erosions by about 87%. Two 5 min of coronary artery or hepatic artery plus portal vein occlusion resulted in similar significant reduction (by about 80%) in gastric lesion area caused by standard ischemia–reperfusion as compared to that achieved with gastric preconditioning. The gastric blood flow in the intact stomach averaged 51±4 ml/min/100 g (taken as 100%) and this was significantly reduced (by about 40%) at the end of 3 h of gastric reperfusion.

Gastric preconditioning by itself produced a significant increase (by about 22%) in the gastric blood flow as compared to that in sham-operated control rats not exposed to ischemia–reperfusion. Cardiac and hepatic preconditioning by themselves significantly raised the gastric blood flow by about 19% and 23%, respectively, as compared to that is

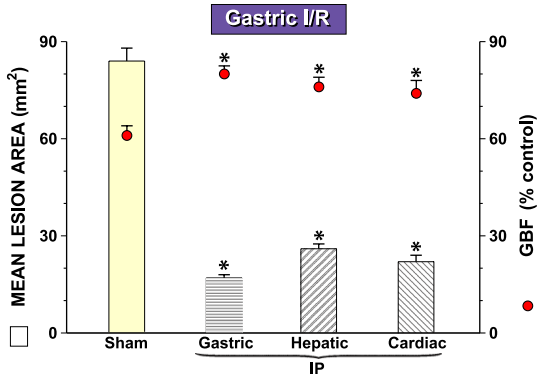

Fig. 1. Mean area of gastric lesions (columns) and gastric blood flow (GBF) (lines) in the gastric mucosa of rats pretreated with sham (control) or gastric, hepatic and cardiac preconditioning (IP) and then exposed to 30 min of gastric ischemia followed by 3 h of reperfusion. Results are mean±S.E.M. of six to eight rats. Asterisk indicates a significant change as compared with the value obtained in sham-control animals.

sham-operated animals not exposed to ischemia–reperfusion. Gastric preconditioning increased significantly the gastric blood flow by about 35% as compared to that recorded in sham-operated controls exposed to standard ischemia–reperfusion. Cardiac or hepatic preconditioning also produced significant rise in the gastric blood flow and this increase was not significantly different from that obtained in gastric mucosa subjected to two brief episodes of gastric ischemia followed by prolonged ischemia–reperfusion.

3.2. Effect of non-selective and selective inhibitors of cyclooxygenase-1 and cyclooxygenase-2 on the protection induced by gastric preconditioning and the changes in the gastric blood flow and prostaglandin E_2 generation in the gastric mucosa

As shown in Fig. 2, gastric preconditioning significantly attenuated the ischemia–reperfusion-induced gastric lesions and significantly raised the gastric blood flow to the extent similar as presented in Fig. 1. In rats treated with indomethacin or SC-560 and then exposed to standard ischemia–reperfusion, the area of erosions caused by this ischemia–reperfusion increased significantly above that recorded in vehicle-treated animals and this was accompanied by the significant fall in the gastric blood flow as compared to the respective values obtained in vehicle-pretreated (sham) rats exposed to ischemia–reperfusion. Pretreatment with rofecoxib also resulted in a significant increase in the area of ischemia–reperfusion-induced gastric lesions and this was accompanied by a marked fall in the gastric blood flow similar to that attained in animals treated with indomethacin or SC-560. The attenuation of the area of gastric lesions and of the accompanying increase in the gastric blood flow induced by gastric preconditioning or that caused by a brief ischemia of remote organs such as heart or liver were significantly reduced by the pretreatment with each cyclooxygenase inhibitor tested (Fig. 2).

Gastric, hepatic or cardiac preconditioning produced significant increase in the generation of prostaglandin E_2 in the gastric mucosa as compared to that measured in gastric mucosa of sham-operated (control) animals not exposed to ischemia–reperfusion (Table 1). Administration of indomethacin or SC-560 significantly decreased the prostaglandin E_2 generation, whereas rofecoxib failed to affect this generation in rats not exposed to ischemia–reperfusion. The exposure of gastric mucosa to standard ischemia–reperfusion that caused gastric lesions produced a significant fall (by about 50%) of the prostaglandin E_2 generation as compared to the values recorded in the sham (control) gastric mucosa (Table 1). The administration of indomethacin (5 mg/kg i.p.) that suppressed significantly mucosal generation of prostaglandin E_2 by about 75% increased significantly the mean area of ischemia–reperfusion-induced lesions and this effect was accompanied by a significant fall in the gastric blood flow (Fig. 2; Table 1).

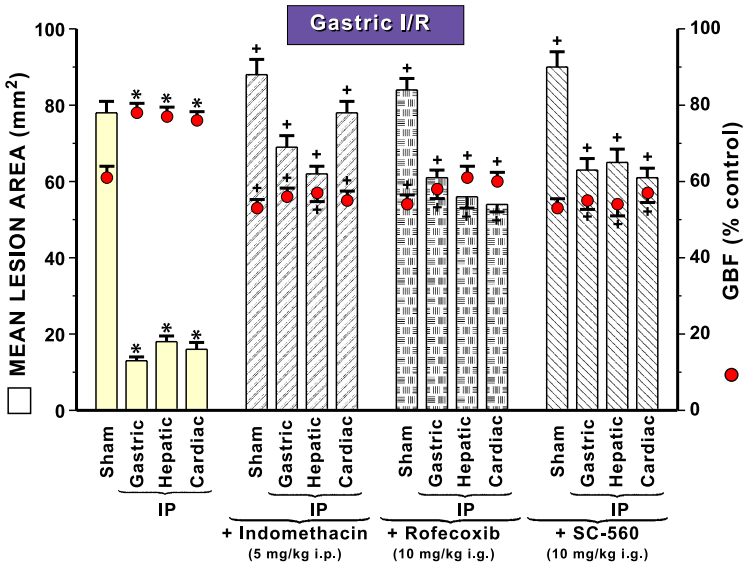

Fig. 2. Effect of sham (control) or brief ischemic preconditioning of the stomach, liver or heart (IP) with or without pretreatment with indomethacin (5 mg/kg i.p.), rofecoxib (10 mg/kg i.g.) and SC-560 (10 mg/kg i.g.) on the area of gastric lesions (columns) and accompanying changes in the GBF (lines) induced by the exposure to prolonged ischemia–reperfusion (I/R). Results are mean±S.E.M. of six to eight 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 treatment with cyclooxygenase inhibitors.

Pretreatment with SC-560, which also augmented significantly the area of gastric lesions induced by ischemia–reperfusion, produced a significant fall in the prostaglandin E_2 generation. When rofecoxib was applied i.g. in a dose of 10 mg/kg, the significant increase in gastric lesions was observed along with a significant inhibition in the mucosal prostaglandin E_2 generation (Fig. 2; Table 1).

Table 1
Effect of sham (control) and gastric, hepatic and cardiac ischemic preconditioning (IP) without or with the pretreatment with indomethacin (5 mg/kg i.p.), SC-560 (10 mg/kg i.g) and rofecoxib (10 mg/kg i.g.), on the mucosal generation of prostaglandin E_2 in gastric mucosa exposed to standard ischemia–reperfusion

Type of test	Prostaglandin E_2 generation (ng/g)		
	Gastric IP	Cardiac IP	Hepatic IP
Without ischemia–reperfusion			
Sham	134±14	138±12	129±9
IP	154±10[a]	159±8[a]	148±11[a]
Indomethacin	33±4[a]	28±6[a]	38±3[a]
SC-560	104±9[a]	110±5[a]	117±8[a]
Rofecoxib	128±13	122±9	125±7
With ischemia–reperfusion			
Sham	68±8[a]	77±6[a]	62±4[a]
IP	118±6[b]	125±9[b]	108±4[b]
Indomethacin+IP	18±3[b]	25±5[b]	21±6[b]
SC-560+IP	55±4[c]	64±5[c]	59±8[c]
Rofecoxib+IP	64±5[c]	58±3[c]	69±7[c]

Results are mean±S.E.M. of 8–10 rats.

[a] Indicates a significant change as compared to the value obtained in sham (control) gastric mucosa.

[b] Indicates a significant change as compared to the value obtained in gastric mucosa of animals exposed to I/R without IP.

[c] Indicates a significant change as compared to the respective value obtained in gastric mucosa not exposed to I/R.

As shown in Fig. 3, the area of gastric lesions measured after ischemia–reperfusion preceded by preconditioning was significantly higher and the gastric blood flow was significantly lower in this series of experiments in rats pretreated with indomethacin (5 mg/kg i.p.), rofecoxib (10 mg/kg i.g.) and SC-560 (10 mg/kg i.g.) applied 30 min before the preconditioning as compared to those recorded in preconditioned animals without cyclooxygenase-1 and cyclooxygenase-2 inhibitors. Addition of prostaglandin E_2 (1 μg/kg i.g.), which by itself failed to influence significantly the ischemia–reperfusion lesions (data not shown), attenuated significantly the enhancement in area of these lesions and reversed the accompanying fall in gastric blood flow induced by indomethacin, SC-560 or rofecoxib (Fig. 3).

3.3. Effect of capsaicin denervation on gastric lesions induced by standard ischemia–reperfusion, gastric blood flow and mucosal generation of prostaglandin E_2 in rats with or without preconditioning

The results of capsaicin deactivation of sensory nerves with or without concurrent treatment with CGRP on gastric lesions induced by standard ischemia–reperfusion with or without gastric, cardiac or hepatic preconditioning and accompanying changes in the gastric blood flow and mucosal generation of prostaglandin E_2 are shown in Fig. 4. Capsaicin denervation increased significantly the ischemia–reperfusion-induced gastric lesions and this effect was accompanied by a small but significant fall in the gastric blood flow and by significant decrease in the prostaglandin

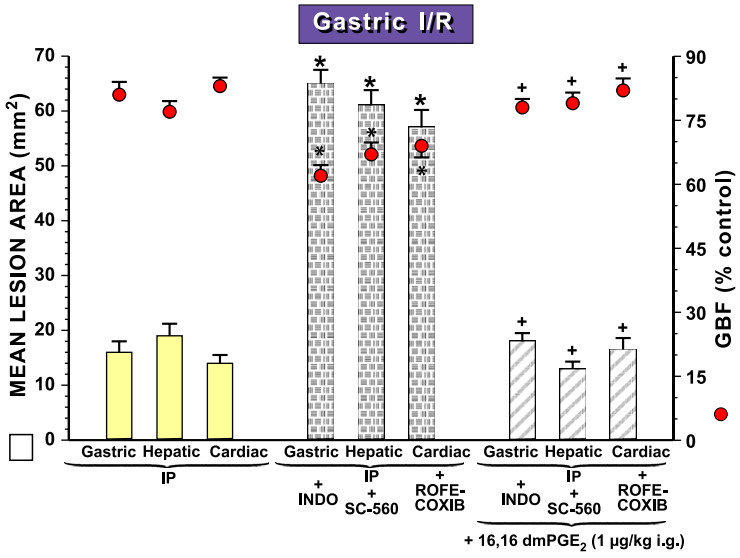

Fig. 3. Effect of gastric, hepatic and cardiac ischemic preconditioning (IP) with or without pretreatment with indomethacin (5 mg/kg i.p.), rofecoxib (10 mg/kg i.g.) and SC-560 (10 mg/kg i.g.) administered with or without addition of 16,16 dm prostaglandin E_2 (PGE_2 1 μg/kg i.g.) on the area of gastric lesions (columns) and accompanying changes in the GBF (lines) induced by the exposure to prolonged ischemia–reperfusion (I/R). Results are mean±S.E.M. of six to eight rats. Asterisk indicates a significant change as compared with the value obtained in rats without the pretreatment with cyclooxygenase inhibitors. Cross indicates a significant change as compared to the value obtained in rats without prostaglandin E_2 administration.

E_2 generation in the gastric mucosa. The pretreatment with gastric, hepatic or cardiac preconditioning before standard ischemia–reperfusion caused usual significant decrease in the area of gastric lesions and the rise in the gastric blood flow and prostaglandin E_2 generation in rats with intact sensory nerves and these effects were significantly attenuated in rats with sensory nerve deactivation by pretreatment with capsaicin (Fig. 4).

Addition of CGRP to capsaicin denervation, which by itself significantly reduced ischemia–reperfusion-induced gastric lesions, almost completely restored the protective effect and accompanying increase in the gastric blood flow and mucosal generation of prostaglandin E_2 evoked by gastric, cardiac or hepatic preconditioning against mucosal damage provoked by ischemia–reperfusion (Fig. 4).

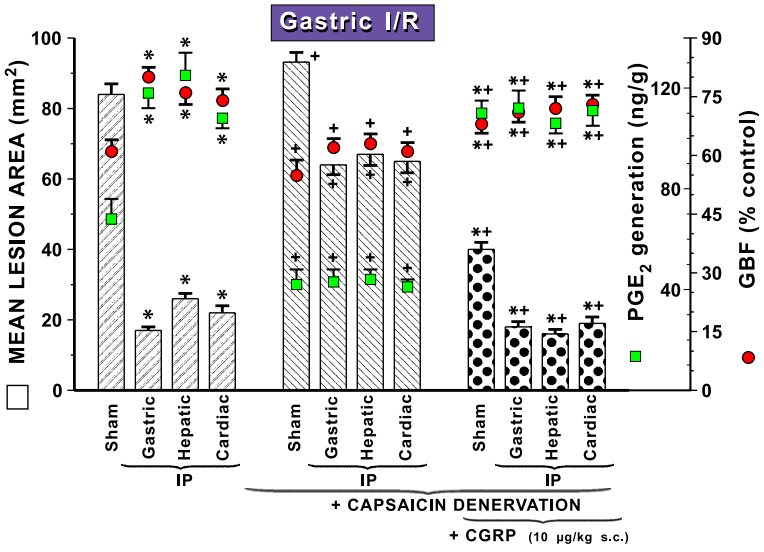

Fig. 4. Effect of sham (control) or gastric, hepatic and cardiac ischemic preconditioning (IP) in rats with intact or capsaicin-deactivated sensory nerves with or without addition of calcitonin gene-related peptide (CGRP, 10 μg/kg s.c) on the area of gastric lesions (columns) and accompanying changes in the GBF (lines) induced by the exposure to prolonged ischemia–reperfusion (I/R). Results are mean±S.E.M. of six to eight 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. Asterisk and cross indicate a significant change as compared with the value obtained in capsaicin-denervated rats without CGRP administration.

Table 2
Effect of sham (control) and gastric, hepatic or cardiac ischemic preconditioning (IP) on the plasma interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) levels in rats exposed to prolonged ischemia–reperfusion (I/R)

Type of test	IL-1β (pmol/ml)	TNF-α (pmol/ml)
Intact	3.5±0.4	1.3±0.2
Sham+I/R	135±12[a]	15±2.8[a]
Gastric IP+I/R	68±5[b]	5.3±1.6[b]
Hepatic IP+I/R	72±3[b]	4.7±1.3[b]
Cardiac IP+I/R	56±7[b]	6.5±2.2[b]

Results are mean±S.E.M. of 8–10 rats.
[a] Indicates a significant change as compared to the value obtained in intact gastric mucosa.
[b] Indicates a significant change as compared to the value obtained in gastric mucosa of animals exposed to sham (control) plus I/R without IP.

3.4. Plasma levels of interleukin-1β and TNF-α in ischemia–reperfusion rats with or without gastric and remote preconditioning

The results of the measurement of plasma interleukin-1β and TNF-α levels in ischemia–reperfusion rats with and without pretreatment with gastric, cardiac or hepatic preconditioning are presented in Table 2. Plasma interleukin-1β and TNF-α concentrations were negligible in the sham-operated animals but rose significantly in ischemia–reperfusion rats not subjected to gastric, cardiac or hepatic preconditioning. Gastric, cardiac or hepatic preconditioning, which by themselves failed to influence significantly plasma interleukin-1β and TNF-α levels in rats not exposed to ischemia–reperfusion (not shown), significantly decreased the plasma level of these cytokines as compared to their respective values in rats exposed to standard ischemia–reperfusion without gastric and remote preconditioning (Table 2).

3.5. Expression of mRNAs for cyclooxygenase-1, cyclooxygenase-2, interleukin-1β and TNF-α by reverse transcriptase-polymerase chain reaction in gastric mucosa exposed to ischemia–reperfusion lesions with or without gastric, cardiac or hepatic preconditioning

Figs. 5A–C and 6A–C show expression of β-actin, cyclooxygenase-1 and cyclooxygenase-2 mRNAs in the intact gastric mucosa of sham-operated control and those subjected to gastric, cardiac or hepatic preconditioning without or with the exposure to ischemia–reperfusion. The expression of β-actin mRNA was well-preserved in the mucosal samples taken both from intact mucosa and those taken from the gastric mucosa of animals exposed to ischemia–reperfusion with or without short ischemia of the gastric mucosa and that of remote organs such as liver or heart (Figs. 5C and 6C; left panels). As shown in Figs. 5A and 6A, the cyclooxygenese-1 mRNA was well detected in the intact gastric mucosa and that exposed to gastric, cardiac or hepatic preconditioning applied alone or in that subjected to gastric and remote preconditioning followed by ischemia–reperfusion. The ratio of mRNA cyclooxygenase-1

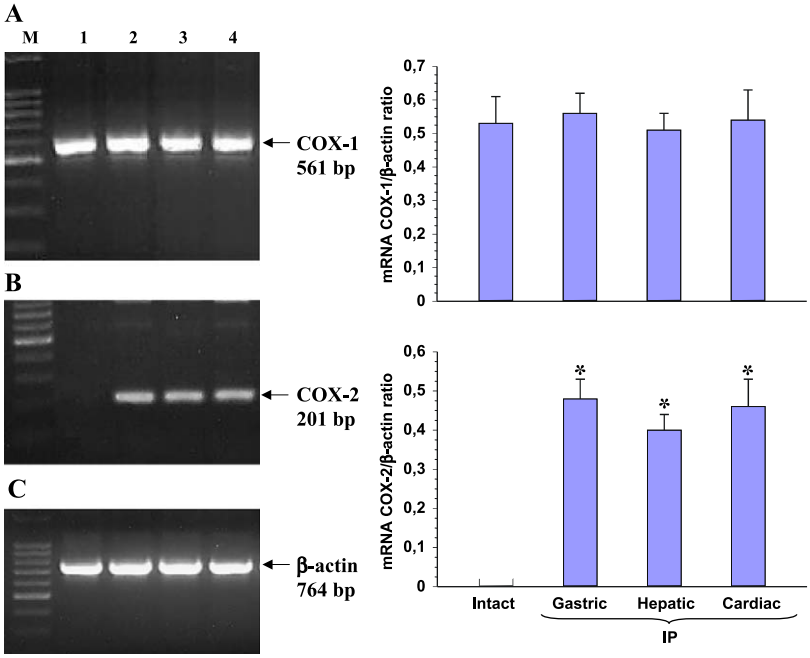

Fig. 5. Messenger RNA expression for cyclooxygenase (COX)-1, cyclooxygenase(COX)-2 and β-actin (left panel) and assessment of mucosal gene expression for cyclooxygenase-1 and cyclooxygenase-2 by the intensity of cyclooxygenase-1, cyclooxygenase-2 mRNA/β-actin mRNA ratio in intact gastric mucosa (lane 1), and in that subjected to gastric, hepatic or cardiac ischemic preconditioning (IP) without ischemia–reperfusion (lanes 2–4). M—size marker DNA, arrow—expected PCR product (bp). Asterisk indicates a significant change as compared with the value obtained in intact gastric mucosa.

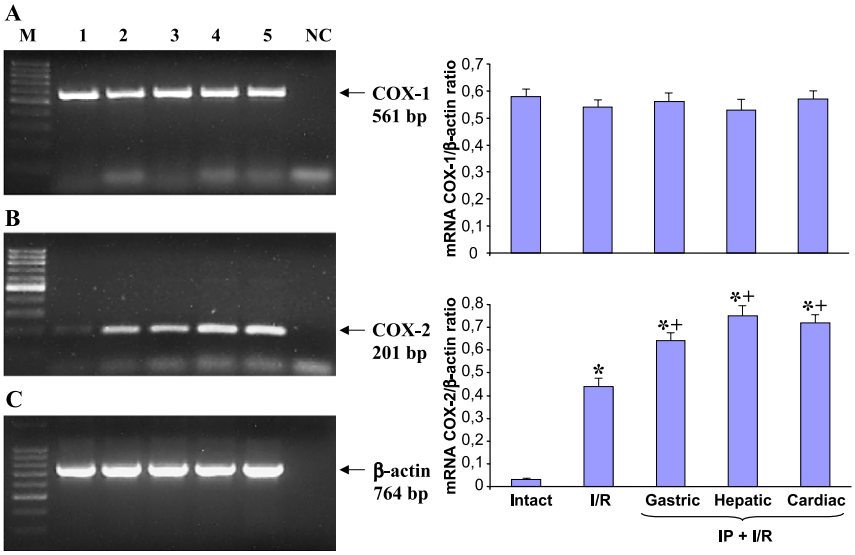

Fig. 6. Messenger RNA expression for cyclooxygenase (COX)-1, cyclooxygenase(COX)-2 and β-actin (left panel) and assessment of mucosal gene expression for cyclooxygenase-1 and cyclooxygenase-2 by the intensity of cyclooxygenase-1, cyclooxygenase-2 mRNA/β-actin mRNA ratio in intact gastric mucosa (lane 1), sham plus ischemia–reperfusion (I/R) (lane 2), gastric, hepatic and cardiac ischemic preconditioning (IP) plus prolonged I/R (lanes 3–5). M—size marker DNA, arrow—expected PCR product (bp). Asterisk indicates a significant change as compared with the value obtained in intact gastric mucosa. Asterisk and cross indicate a significant change as compared with the value obtained in animals exposed to sham plus I/R.

over *β*-actin revealed that the expression of cyclooxygenase-1 mRNA was similar in gastric mucosa taken from rats with ischemia–reperfusion alone and those pretreated with gastric, cardiac and hepatic preconditioning with or without the exposure to this ischemia–reperfusion (Figs. 5 and 6, right panels). In contrast, the signal for cyclooxygenase-2 mRNA was barely detectable in intact animals but has been traced in rats exposed to ischemia–reperfusion and in those pretreated with preconditioning of stomach, heart or liver without or with the exposure to ischemia–reperfusion (Figs. 5B and 6B; left panels). The ratio of cyclooxygenase-2 mRNA over *β*-actin mRNA revealed that the expression of this enzyme mRNA in gastric mucosa of rats pretreated with gastric, cardiac or hepatic preconditioning was significantly higher than that recorded in sham-operated animals with intact gastric mucosa or in those with ischemia–reperfusion

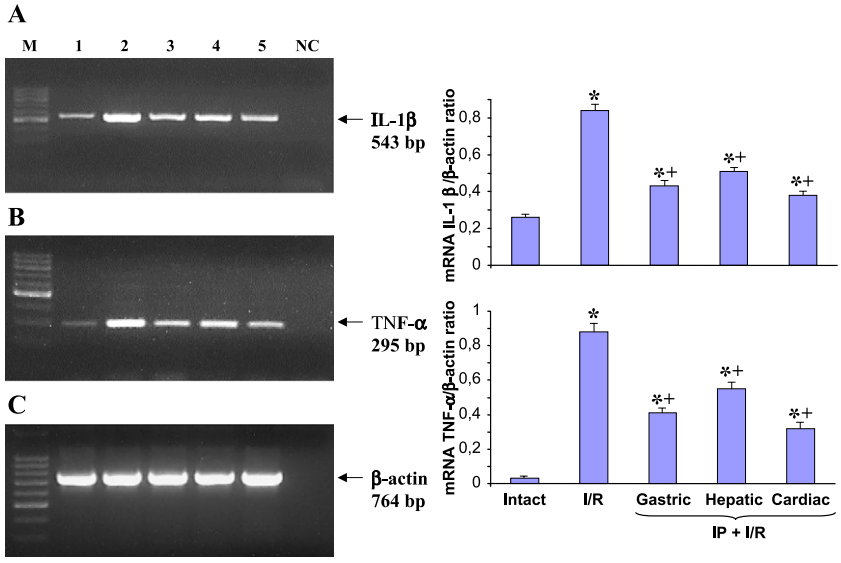

Fig. 7. Messenger RNA expression for β-actin, interleukin-1β and TNF-α (left panel) and assessment of mucosal gene expression for iterleukin-1β and TNF-α by the intensity of interleukin-1β and TNF-α mRNA/β-actin mRNA ratio in intact gastric mucosa (lane 1), sham plus ischemia–reperfusion (I/R) (lane 2), gastric, hepatic and cardiac ischemic preconditioning (IP) plus prolonged I/R (lanes 3–5). M—size marker DNA, arrow—expected PCR product (bp). Asterisk indicates a significant change as compared with the value obtained in intact gastric mucosa. Asterisk and cross indicate a significant change as compared with the value obtained in rats exposed to sham plus I/R.

alone without gastric, cardiac or hepatic preconditioning (Figs. 5 and 6, right panels).

At the mRNA level, the mRNAs for interleukin-1β and TNF-α were barely expressed in sham-operated gastric mucosa but significantly upregulated in the mucosa exposed to standard ischemia–reperfusion without preconditioning (Fig. 7A,B, left panel). Weak signals for these cytokines were recorded in gastric mucosa of animals subjected to gastric, cardiac and hepatic preconditioning prior to ischemia–reperfusion. Ratio mRNA interleukin-1β and TNF-α over β-actin confirmed that the expression of interleukin-1β and TNF-α mRNAs was significantly decreased in gastric mucosa of rats pretreated with gastric, cardiac and hepatic preconditioning prior to ischemia–reperfusion as compared to that obtained in rats exposed to ischemia–reperfusion alone (Fig. 7, right panel).

4. Discussion

This study demonstrates for the first time that preconditioning of the remote organs to the stomach such as heart or liver by brief episodes of ischemia, which by themselves failed to cause gastric damage and produced a small rise in gastric blood flow, exerts potent protective influence on gastric mucosa subjected to prolonged ischemia–reperfusion. To our best knowledge, this is the first demonstration of the gastroprotection phenomenon against ischemia–reperfusion by brief ischemic preconditioning of extragastric organs. Moreover, we confirmed our previous observations that ischemic preconditioning, which has been originally described in various organs including heart, lungs, liver, pancreas and intestine (Murry et al., 1986; Mounsey et al., 1992; Davenpeck et al., 1994; Parratt, 1994; Russell et al., 1996; Ishida et al., 1997; Peralta et al., 1998; Nilsson et al., 2000), could be considered as a powerful intervention in the stomach resulting in a remarkable attenuation of the extent of mucosal damage evoked by the severe ischemia–reperfusion (Konturek et al., 2001; Pajdo et al., 2001). Based on our present results, it is reasonable to assume that remote preconditioning, affording gastroprotection, involves crucial mediators including prostaglandin derived mainly from enhanced cyclooxygenase-2 activity and excessive release of neuropeptides from sensory nerves that appear to play a key role in the mechanism of this protection probably due to rise in the gastric blood flow resulting in vasodilatation. This notion is supported by the fact that gastroprotection and accompanying rise in the gastric blood flow induced by gastric, cardiac or hepatic preconditioning were significantly attenuated by non-selective (indomethacin) and selective cyclooxygenase-1 (SC-560) and cyclooxygenase-2 inhibitor (rofecoxib) and by capsaicin ablating functionally sensory nerves that are known to release NO and various vasodilatatory neuropeptides such as CGRP. Moreover, the concurrent treatment with synthetic prostaglandin E_2 analog to compensate for the deficiency of endogenous prostaglandin, or with exogenous CGRP to replace the neuropeptide lost by deactivation with neurotoxic dose of capsaicin of afferent nerves counteracted the deleterious effects of cyclooxygenase inhibitors and capsaicin-induced denervation in preconditioned gastric mucosa exposed to subsequent ischemia–reperfusion.

Preconditioning can be considered as a general phenomenon in which a tissue is rendered resistant to the deleterious effects of prolonged ischemia–reperfusion by previous exposure to brief periods of vascular occlusion (Ishida et al., 1997). While the beneficial effects of preconditioning were first demonstrated in the myocardium (Murry et al., 1986), it is now evident that such preconditioning also affords protection against post-ischemic damage of skeletal muscle, brain and gastrointestinal organs such as small intestine, pancreas and liver (Murry et al., 1986; Mounsey et al., 1992; Parratt, 1994; Bouchard and Lamontagne, 1996; Tsuruma et al., 1996; Peralta et al., 1998). The mechanism of preconditioning-induced protection has not been fully explained but activation of adenosine A_1 receptors and ATP-sensitive potassium channels in the heart as well as an inhibition of neutrophil activation and emigration though the wall of vessels in the intestine or pancreas were implicated in this phenomenon (Ishida et al., 1997; Lochner et al., 2002). Two types of protection can be afforded by preconditioning, namely an acute and delayed (Wang et al., 2001; Jaeschke, 2003), but little information is available regarding the mechanism of such organ protection induced by preconditioning of remote organs. It has been demonstrated that myocardial protection can be conferred by an earlier brief ischemia of a remote myocardial region which was called preconditioning at the distance or even by brief ischemia of a remote organ, such as the kidney or the mesentery (Gho et al., 1996). Tang et al. (1999) have demonstrated that brief ischemia of the small intestine induces both early and delayed protection against ischemia–reperfusion myocardial injury and that this protective effect depends upon the activation of capsaicin-sensitive nerves. Peralta et al. (1999) have demonstrated that ischemic preconditioning of liver effectively prevented not only the ischemia–reperfusion-induced liver injury and attenuated the release of TNF-α but also ameliorated that to the lungs and this protection was found to involve NO-dependent pathway. All these studies supported the original finding by Gho et al. (1996) that ischemic preconditioning of the remote organs protects the myocardium against infarction as effectively as myocardial preconditioning itself.

According to our best knowledge, no attempts were made to examine the effect of preconditioning of remote organs such as heart or the liver on gastric mucosal damage induced in the stomach by severe ischemia–reperfusion. We found that remote ischemic preconditioning of heart or liver greatly reduced the gastric mucosal lesions caused by severe gastric ischemia–reperfusion

mimicking the gastroprotective effect evoked by brief ischemia episodes of the celiac artery directly supplying the stomach. Moreover, we documented that this gastroprotective effect of remote preconditioning involves the comparable increase in the gastric blood flow and the mucosal generation of prostaglandin E_2 with the magnitude similar to that exhibited by gastric preconditioning itself. The possible mediators of this protection induced by distant preconditioning could be endogenous prostaglandin derived from the enhanced activity of gastric mucosal cyclooxygenase isoforms, especially of cyclooxygenase-2, which was previously implicated in the mechanism of gastric integrity, gastroprotection and ulcer healing due to its upregulation at the edge of chronic gastric ulcers and in the mucosa with ischemia–reperfusion erosions progressing into deeper ulcerations (Konturek et al., 1992; Eberhart and Dubois, 1995; Mizuno et al., 1997; Brzozowski et al., 1999). Previous evidence indicated that certain prostaglandin such as prostacyclin, which is released from ischemic myocardium, might limit the extent of heart infarct and attenuate ventricular arrhythmia (Murry et al., 1986; Parratt, 1994). Moreover, inhibition of cyclooxygenase-2 activity by NS-398, a selective inhibitor of cyclooxygenase-2, prevented the protective effect of ischemic preconditioning in dog and rabbit myocardium (Shinmura et al., 2000). Our study are in keeping with these findings by showing directly gene overexpression of cyclooxygenase-2 in the gastric mucosa following gastric, cardiac and hepatic preconditioning applied alone or combined with severe ischemia–reperfusion while cyclooxygenase-1 mRNA remained unchanged. Moreover, the suppression of the prostaglandin biosynthesis by non-selective (indomethacin) and highly selective cyclooxygenase-1 (SC-560) or cyclooxygenase-2 (rofecoxib) inhibitors markedly attenuated the protective and hyperemic effects of gastric as well as cardiac and hepatic preconditioning. Furthermore, minute amounts of synthetic prostaglandin E_2 analogue added to these cyclooxygenase inhibitors restored the protective and hyperemic effects of preconditioning in gastric mucosa, thus reinforcing the notion that endogenous prostaglandin, produced in excessive amounts by cyclooxygenase-2, plays an important role in the mechanism of remote as well as close gastric preconditioning.

It is possible that the enhanced expression of cyclooxygenase-2 was triggered by the proinflammatory cytokines, interleukin-1β and TNF-α, as proposed by Takahashi et al. (1998) and Shigeta et al. (1998). These cytokines were implicated in the induction of inflammation, injury and carcinogenesis in a variety of tissues including the gastric mucosa (Le and Vilcek, 1987; Diamond and Pesek, 1991; Troost et al., 2003) and in the mechanism of ischemia–reperfusion injury of gastric mucosa progressing into gastric ulcer (Brzozowski et al., 2000; Konturek et al., 2000). We confirmed our own observations (Brzozowski et al., 1999, 2000) that both expression and release of these cytokines are increased in animals exposed to gastric ischemia–reperfusion and we found for the first time that these effects were attenuated in rats exposed to cardiac, hepatic and gastric preconditioning resulting in the gastroprotection against ischemia reperfusion injury and mucosal hyperemia. Interestingly, sham-operated control rats failed to exhibit increased expression and release of interleukin-1β and TNF-α ruling out the possibility that surgical procedure by itself could alter significantly the cytokine generation. Thus, we conclude that the suppression of proinflammatory cytokines such as interleukin-1β and TNF-α expression and release could contribute to the protective effect of remote and gastric preconditioning in the stomach.

It is of interest that the reversal of protective and hyperemic effects as well as accompanying increase in gastric mucosal generation of prostaglandin E_2 of remote or close gastric preconditioning against severe gastric ischemia–reperfusion was observed in rats with capsaicin-induced deactivation of sensory nerves, suggesting the involvement of the brain–gut axis in these gastroprotective effects of both preconditioning (Pawlik et al., 2001). CGRP released from sensory nerves and endogenous prostaglandins appear to cooperate in the beneficial effects of short ischemic episodes against severe lesions caused by prolonged ischemia–reperfusion. This notion is supported by our finding that gastroprotective and hyperemic effects of gastric and remote preconditioning and accompanying rise in the gastric mucosal generation of prostaglandin E_2 were markedly attenuated by capsaicin denervation. Moreover, the co-administration of CGRP, added to remote and gastric preconditioning in rats with functionally ablated sensory nerves almost completely restored the gastroprotective and hyperemic effects of these preconditionings and the capability of preconditioned gastric mucosa to generate of prostaglandin E_2. This is in keeping with original observation of Ferdinandy et al. (1997) and Tang et al. (1999), suggesting that capsaicin-sensitive afferent nerves are involved in the cardiac preconditioning, and by Xiao et al. (2001), that cardioprotection achieved by intestinal preconditioning is mediated by endogenous CGRP. The possibility cannot be excluded that gastroprotection induced by gastric and remote preconditioning could be attributable to sensitization by prostaglandins of sensory nerves releasing of CGRP. This is consistent with the recent hypothesis that endogenous prostaglandins contribute to the adaptive cytoprotection against gastric injury induced by mild irritant via the enhancement of CGRP released from afferent sensory nerves (Boku et al., 2001; Konturek et al., 1982).

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