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Gastric preconditioning induced by short ischemia: the role of prostaglandins, nitric oxide and adenosine

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SUMMARY

Background: *Various organs including heart, kidneys, liver or brain respond to brief exposures to ischemia with an increased resistance to severe ischemia and this phenomenon is called 'preconditioning'. No study so for has been undertaken to check whether such short, repeated gastric ischemic episodes protect gastric mucosa against the damage caused by subsequent prolonged ischemia-reperfusion or necrotizing substances.*

Material and Methods: *In this study, cyclooxygenase (COX)-1, COX-2, nitric oxide (NO) and adenosine receptors inhibitors were used to determine the possible involvement of endogenous prostaglandin, NO and adenosine in the mechanism of gastric preconditioning. This ischemic preconditioning was induced by short episodes of occlusion of celiac artery from 1 to 5 times, for 5 min each applied 30 min before prolonged (30 min) ischemia followed by 3 h of reperfusion (I/R) or 30 min before topical application of strong mucosal irritants such as 100% ethanol, 25% NaCl or 80 mM taurocholate.*

Results: *Exposure to regular I/R produced numerous gastric lesions and significant fall in the gastric blood flow and PGE_2 generation. Short (5 min) ischemic episodes even induced several times (1-5 times) by itself failed to cause any gastric lesions but significantly attenuated those produced by I/R and this protective effect reached maximum with two 5 min ischemic episodes and this preconditioning was considered as standard. The protective effects of standard ischemic preconditioning against gastric lesions induced by I/R was accompanied by a reversal of the fall in the gastric blood flow and PGE_2 generation and resembled those induced by classic gastric mild irritants such as 20% ethanol, 5% NaCl and 5 mM taurocholate. These protective and hyperemic effects of standard preconditioning, lasted up to 6-8 h, and were significantly attenuated by pretreatment with specific COX-1 and COX-2 inhibitors such as Vioxx (5 mg/kg i.g.) and resveratrol (10 mg/kg i.g.) that failed to affect PGE_2 generation in intact gastric mucosa but attenuated significantly that in preconditioned gastric mucosa. Non-specific COX-inhibitor indomethacin (5 mg/kg i.p.), that suppressed the PGE_2 generation by ~90% and non-specific NO synthase inhibitor L-NNA (20 mg/kg i. p.), that significantly suppressed NO production, significantly inhibited the protection and the rise in GBF induced by standard preconditioning and these effects were restored by addition of 16,16 dm PGE_2 (1 μg/kg i.g.) or L--arginine (200 mg/kg i.g.), a substrate for NO-synthase, to indomethacin or L-NAME, respectively. Pretreatment with adenosine (10 mg/kg i.g.) also reduced the lesions induced by I/R and increased the gastric blood flow with the extent similar to that observed with standard ischemic preconditioning, while an antagonist of adenosine receptors, 8-phenyl theophylline (SPT, 10 mg/kg i.g.) attenuated significantly the gastroprotection afforded by the preconditioning. Gene expression of COX-1 but not COX-2 was detected by RT-PCR in intact gastric mucosa and in that exposed to I/R with or without ischemic preconditioning, whereas COX-2 was overexpressed only in preconditioned mucosa.*

Conclusions: *1) gastric ischemic preconditioning represents one of the most powerful protective intervention against the mucosal damage induced by severe I/R as well as by topical mucosal irritants in the stomach; 2) this protection, involving several mediators such as PG derived from COX-1 and COX-2, NO originating from NO-synthase and adenosine, appear to play a key mechanism of gastric ischemic preconditioning.*

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BACKGROUND

Ischemia preconditioning refers to a phenomenon in which a tissue is rendered resistant to the deleterious effect of prolonged severe ischemia by previous exposures to brief vascular occlusions [1]. This protective effect 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 and skeletal muscle induced by subsequent exposure to severe ischemia in a variety of species [3–6], but the mechanism of this organ protection by such ischemic preconditioning has not been fully clarified.

The protective activity of ischemic preconditioning is best documented phenomenon in the heart where repeated short episodes of coronary occlusion were shown to prevent lethal injury of the myocardium induced by subsequent long-term and severe I/R [7,8]. 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 [9,10]. This indicates that ischemic preconditioning also exists in the gut, possibly preventing the mesenteric microvascular barrier dysfunction and activation of excessive amount of NO in the intestine [9–11].

The mechanism of ischemic preconditioning remains unclear but adenosine that is produced during the ischemic preconditioning was proposed to act as an initiator of this preconditioning in different organs, because of beneficial effect of adenosine in attenuating the injury caused by severe I/R and an evidence that protective effects of ischemic preconditioning can be reversed by adenosine receptor antagonists [6,12].

The question remains whether similar protective effect of ischemic preconditioning can also be observed in the gastric mucosa subjected to longer I/R or to strong mucosal irritants and if so which mechanism is involved in this preconditioning response. Similar protective effects so called adaptive cytoprotection, were originally revealed in the stomach by Robert et al, [13] and confirmed by our group [14] more than two decades ago by showing the protective action of certain mild irritants such as 20% ethanol, 5% NaCl or 5 mM taurocholate against the damage induced by these agents applied intragastrically in large mucosal necrotizing concentrations. This action of mild irritants has been predominantly attributed to the protective effects of endogenous prostaglandin but then besides prostaglandin many other protective factors such as nonprotein sulfhydryl compounds NO, sensory nerves, calcitonin gene related peptide (CGRP) and have been implicated in this phenomenon [15–18]. The oldest mediator of gastroprotection, prostaglandin were found to originate from at least two cyclooxygenases (COX), one constitutive (cyclooxygenase-1) playing role in physiological of mucosal homeostasis and another, inducible (cyclooxygenase-2) isoform that is expressed at a site of inflammation [19,20], but the contribution of either isoform of cyclooxygenases in gastric preconditioning has not been explored.

This study was designed to determine whether ischemic preconditioning exists in the stomach and if so to elucidate the contribution of endogenous prostaglandins and NO to gastroprotection against I/R induced by standard ischemic preconditioning. We also attempted to explore the involvement of adenosine in the mechanism of ischemic preconditioning and to assess the mucosal gene and protein expression of COX-1 and COX-2 in gastric mucosa subjected to ischemic preconditioning with or without prolonged I/R.

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.

Production of gastric lesions induced by ischemia-reperfusion

I/R-induced erosions were produced in 120 rats by the method originally proposed by Wada et al [21]. 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-ischemic preconditioning) was applied 30 min before subsequent exposure to longer (regular) 30 min of 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 the celiac artery was only slightly manipulated but not occluded. First, we attempted to deter-

mine the effect of various time periods of gastric ischemic preconditioning on the lesions induced by regular I/R. For this purpose, rats were preconditioned with single episode of gastric preconditioning ranging from 37 up to 300 s before the exposure to 30 min of ischemia followed by 3 h of reperfusion. Second, we wanted to know whether the increasing number of short ischemic episodes affects the lesions induced by I/R. For this purpose gastric mucosa was pretreated with 1 to 5 episodes of short ischemia (5 min each) before the exposure to regular I/R. In another group of rats, the duration of protective effect of standard ischemic preconditioning (2 × 5 min occlusion) against the gastric erosions caused by regular I/R was studied. The duration of the preconditioning effect was examined in rats pretreated with standard (2 × 5 min occlusion) ischemic preconditioning followed 1h, 2h, 4h, 8h or 12 h later by regular I/R.

Effect of suppression of COX-1 and COX-2 activity on gastric ischemic preconditioning

In separate group of rats, the pretreatment with COX inhibitors was employed 30 min prior to gastric preconditioning followed by 3 h of I/R in order to determine whether suppression of non-selective or selective inhibitors of COX-1 and COX-2 influences the protective action of the preconditioning.

Several groups of rats, each consisting of 6-8 animals, were given 30 min before gastric preconditioning one of the following treatments: 1) vehicle (saline), 2) resveratrol (1,3-Benzenediol,5-[2-(4-hydroxyphenyl) ethenyl], 10 mg/kg i.g.), a selective COX-1 inhibitor [22]; 3) Vioxx (5 mg/kg i.g), the highly selective COX-2 inhibitor [23]; and 4) indomethacin (5 mg/kg i.p.), a non-selective cyclooxygenase inhibitor [24]. At the dose used in present study, indomethacin has been shown previously to inhibit gastric prostaglandin E_2 generation capability by ~ 90 % without causing by itself any mucosal damage [25]. The dose of Vioxx was selected on the basis of previous studies showing that this agent failed to affect the generation of prostaglandin E_2 (PGE_2) in intact gastric mucosa but inhibited significantly the gastric PGE_2 production in ulcerated gastric mucosa [23]. Resveratrol (Cayman Chemical Co, Ann Arbor, Michigan, USA) 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. Vioxx (Merck Sharp & Dohme) 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 [23]. Resveratrol and Vioxx were used in a dose (10 mg/kg and 5 mg/kg i.g., respectively) that were shown by our group to inhibit the PGE_2 generation in the gastric mucosa injured by I/R [23]. 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 standard ischemic preconditioning and then to I/R with or without treatment with COX-1 and COX-2 inhibitors, the prostaglandin deficit were replaced 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 I/R and accompanying fall in gastric blood flow (data not shown). For this purpose, 16,16 dimethyl prostaglandin E2 analog was administered together with each COX-1 or COX-2 inhibitor starting 30 min prior to standard ischemic preconditioning followed by 3 h of I/R.

The area of gastric lesions was determined using a planimeter (Morphomat, Carl Zeiss, Berlin, Germany) under blinded conditions according to the method described previously [26].

Involvement of NO in the protective effect of gastric preconditioning

The implication of NO in the effect of gastric preconditioning on damage induced by I/R was determined by three ways; 1) by the using N^G-nitro-L-arginine (L-NNA) applied i.g. in a dose of 20 mg/kg to suppress non-specifically the activity of NOS [27]; 2) by the indirect measurement of NOS product i.e. NO in gastric lumen [28]; and 3) by addition to L-NNA of L-arginine, a substrate for NOS or D-arginine, which is not a substrate for NO [29]. The rats with gastric lesions induced by regular I/R were pretreated either with: 1) sham operation or standard ischemic preconditioning (occlusion of celiac artery twice for 5 min) alone; 2) L-NNA (20 mg/kg i.g.) with or without the preconditioning; 3) L-arginine (200 mg/kg i.g.) plus L-NNA (20 mg/kg i.g.) combined with the preconditioning, and finally; 4) D-arginine (200 mg/kg i.g.) plus L-NNA (20 mg/kg i.g.) combined with the preconditioning.

The luminal concentration of NO was quantified indirectly as nitrate (NO_3^-) and nitrite (NO_2^-) levels in

the gastric contents using the nitrate/nitrite kit purchased from Cayman Lab, Michigan, USA as described in details before [29]. This method is based on the Griess reaction and generation of chromophore absorbing at 595 nm, according to the original procedure reported previously [30]. Since NO released by epithelial cells into the gastric lumen is quickly transformed into NO_3^- and NO_2^- [28], we measured photometrically the sum of both these products of NOS as an index of production of NO by the enzyme in the gastric mucosa. For this purpose, the gastric content was aspirated just before the removal of the stomach following the i.g. injection of 1 ml of saline to wash out the luminal content. After centrifugation for 10 min at 3000 rpm, the samples were mixed with Griess reagent from the commercially available kit. In all tests including gastric preconditioning with or without the combination with L-NNA and L-arginine or D-arginine, the GBF was measured in the oxyntic mucosa in each group of animals in similar manner as mentioned before and expressed as the percent control value recorded in vehicle--treated gastric mucosa.

Implication of adenosine in ischemic gastric preconditioning

The involvement of adenosine in the mediating of the effect of preconditioning on the gastric mucosa was determined by two ways: 1) by pretreatment with 8-p-sulphophenyl theophylline (SPT) at a dose (10 mg/kg i.g.), that was reported to inhibit adenosine receptors and to attenuate the effect of ischemic preconditioning on the heart infarct size [31], and 2) by the application of exogenous adenosine (10 mg/kg i.g.) to check whether pretreatment with exogenous adenosine can protect the gastric mucosa lesions induced by regular I/R.

Measurement of gastric blood flow (GBF)

At the termination of each experiment, the gastric blood flow (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 gastric blood flow 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/100 g--min) of these measurements were calculated and expressed as percent changes from those recorded in control animals treated with vehicle.

Determination of mucosal generation of PGE_2

In groups of rats exposed to standard ischemic preconditioning followed by regular I/R without or with pretreatment with COX-inhibitors, the mucosal samples form the oxyntic gland area were taken by biopsy (about 200 mg) from gastric mucosa without mucosal lesions immediately after the animals were sacrificed to determine the mucosal generation of PGE_2 by radioimmunoassay (RIA) as described previously [25]. 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 RIA. The capability of the mucosa to generate PGE_2 was expressed in nanograms of wet tissue weight.

Expression of COX-1 and COX-2 mRNA transcripts in the gastric mucosa determined by RT-PCR

COX-1 and COX-2 mRNA were determined by RT-PCR in the gastric mucosa of intact rats or those exposed to I/R with or without ischemia preconditioning. Samples of the gastric oxyntic mucosa (about 500 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 [32] using a rapid guanidinum isothiocyanate/phenol chloroform single step extraction kit from Stratagene (Stratagene GmbH, 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 GmbH, Heidelberg, Germany). The primers for COX--1 and COX-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 [33-36]. The COX-1 primer sequences were as follows: up-stream, 5'-AGC CCC TCA TTC ACC CAT CAT TT; downstream, 5'--CAG GGA CGC CTG TTC TAC GG. The expected length of this PCR product was 561 bp. The COX-2 primer sequences were as follows: upstream, 5'-ACA ACA TTC CTT CCT TC; downstream, 5'-CCT TAT

TTC CTT TCA CAC C. The expected length of this PCR product was 201 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.

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 ischemic episodes on the gastric lesions induced by I/R insult and the accompanying changes in the GBF

Figure 1 shows the effects of various time duration lasting from 37 s up to 300 s of single short ischemic episode on gastric lesions and accompanying changes in the gastric blood flow induced by regular I/R. Ischemic episodes shorter than 75 s failed to influence significantly the area of I/R-induced gastric lesions and to affect the gastric blood flow. With prolongation of ischemic episodes up to 150 s or 300 s applied before regular I/R a significant reduction in the area of acute gastric lesions and a significant rise of the gastric blood flow were observed. The short ischemic of 5 min (300 s) that caused reduction of I/R lesions by about 80% was considered as a standard ischemic preconditioning and used in subsequent studies.

Figure 2 shows the effect of various numbers of standard (5 min) ischemic episodes ranging from 1 to 5 on the area of gastric erosions induced by regular I/R. Single standard (5 min) preconditioning episode reduced the area of I/R erosions by about 67%. Increase in number of standard ischemic episodes to two (2 × 5 min occlusion) did not result in any further significant reduction in lesion area caused by regular I/R. The GBF in the intact stomach averaged 53±6 (taken as 100%) and this was significantly reduced (by about 40%) at the end of 3 h of reperfusion that followed 30 min of ischemia. A single standard ischemic episode increased significantly the GBF by about 25% as compared to that recorded in sham-operated controls exposed to regular I/R. Exposure of the gastric mucosa to 2–5 ischemic episodes produced similar rise in the gastric blood flow but this increase was not significantly different than that obtained in animals with single ischemic episode.

Figure 3 shows the duration of the protective effect of standard (2 × 5 min) ischemic preconditioning against regular I/R. This protection followed by the rise in the gastric blood flow was observed starting from 2 h after the beginning of ischemic preconditioning and found to last up to 8 h but after 12 h since the preconditioning such a protective effect and accompanying hyperemia were lost and the area of gastric lesions reached the value not significantly different from that recorded in rats exposed to regular I/R.

As shown in Figure 4, the pretreatment with standard preconditioning resulted in a significant attenuation of gastric lesions induced by regular I/R and in accompanying rise in the GBF similar to that presented in Figure 2. This gastroprotection and the rise in the GBF achieved by a standard preconditioning against gastric lesions evoked by regular I/R were not significantly different from those obtained in animals pretreated with mild irritants such as 20% ethanol, 5% NaCl and 5 mM taurocholate against mucosal damage induced by 100% ethanol, 25% NaCl and 80 mM taurocholate, respectively.

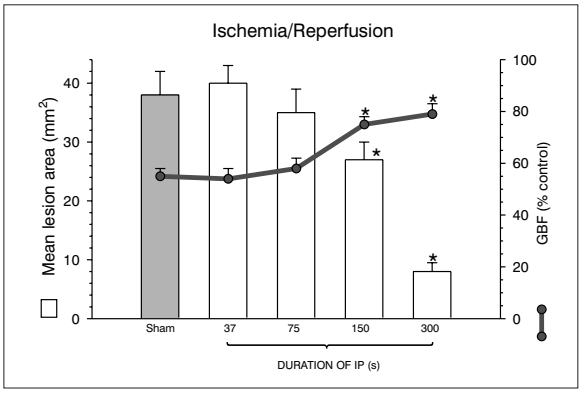

Figure 1. Mean area of gastric lesions and the gastric blood flow (GBF) in the gastric mucosa of rats pretreated with sham (control) or ischemic preconditioning (IP) lasting from 37 up to 300 s and then exposed to 30 min of ischemia followed by 3 h of reperfusion. Results are mean±SEM of 6–8 rats. Asterisk indicates a significant change as compared with the value obtained in sham-control animals.

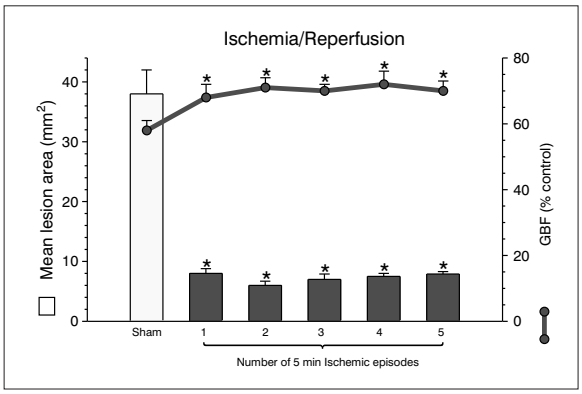

Figure 2. Effect of various numbers of 5 min ischemic episodes on the area of acute gastric lesions and GBF in the gastric mucosa of rats exposed to regular ischemia/reperfusion. Results are mean±SEM of 6–8 rats. Asterisk indicates a significant change as compared with the value obtained in sham-control animals.

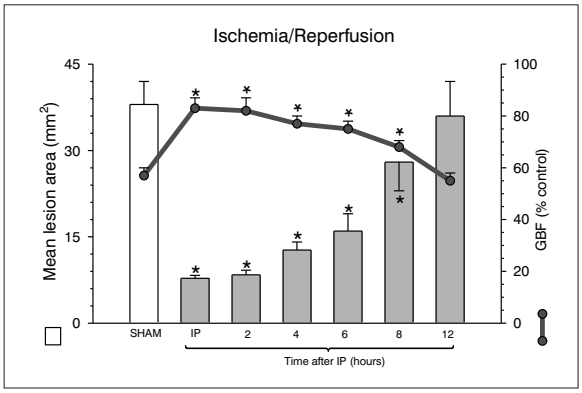

Figure 3. Effect of standard ischemic preconditioning followed 2 h, 4 h, 6 h, 8 h and 12 h later by regular ischemia/reperfusion on the area of gastric lesions and GBF. Results are mean±SEM of 6–8 rats. Asterisk indicates a significant change as compared with the value obtained in sham-operated control rats.

Figure 5 shows that standard preconditioning applied 30 min before regular I/R, 100% ethanol, 25% NaCl or 80 mM taurocholate attenuated significantly the gastric lesions induced by each of these strong irritants applied alone. Standard preconditioning applied 30 min before i.g. application of 100% ethanol, 25% NaCl or 80 mM taurocholate resulted in the significant attenuation of the lesion area and in the rise in GBF as compared to those recorded in sham-operated controls exposed 30 min later to 100% ethanol, 25% NaCl or 80 mM taurocholate (Figure 5). The GBF in preconditioned gastric mucosa followed by intragastric application of necrotizing agents raised to similar extent to those treated with this preconditioning and exposed to regular I/R.

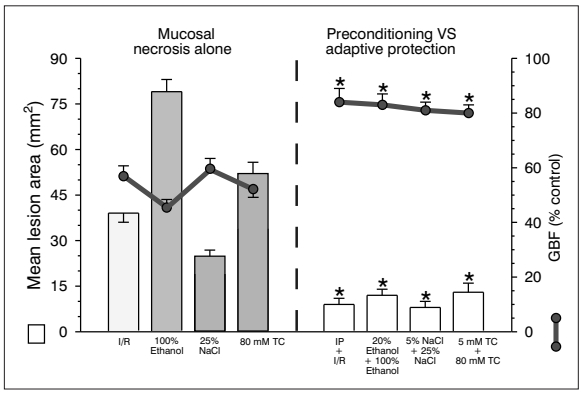

Figure 4. Effect of regular ischemia/reperfusion (I/R), 100% ethanol, 25% NaCl and 80 mM taurocholate (TC) applied alone or pretreated with standard ischemic preconditioning (IP), 20% ethanol, 5% NaCl and 5 mM TC on the area of gastric lesions and accompanying changes in the GBF. Results are mean±SEM of 6–8 rats. Asterisk indicates a significant change as compared with the value obtained in gastric mucosa without IP.

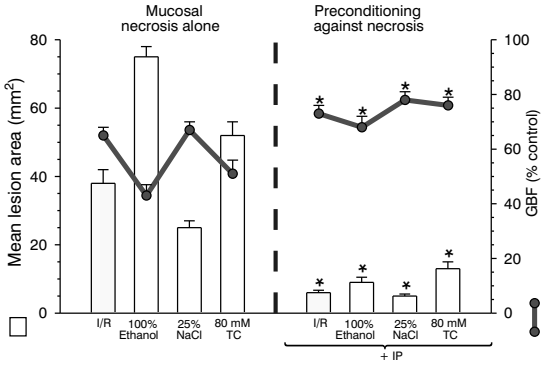

Figure 5. Effect of the pretreatment with standard IP on the area of gastric lesions and accompanying changes in the GBF induced by the exposure to regular I/R, 100% ethanol, 25% NaCl and 80 mM TC. Results are mean±SEM of 6–8 rats. Asterisk indicates a significant change as compared with the value obtained in gastric mucosa without IP.

Effect of non-selective and selective inhibitors of COX-1 and COX-2 on the protection induced by gastric preconditioning and the changes in the GBF and PGE_2 generation in the gastric mucosa

As shown in Figure 6, standard (2 × 5 min) preconditioning attenuated the I/R-induced gastric lesions and raised the GBF to the extent similar as presented in Figs. 2 and 3. Indomethacin, Vioxx and resveratrol by themselves failed to influence significantly the lesions and accompanying fall in the GBF induced by I/R. In rats pretreated with indomethacin and then exposed to standard precondi-

tioning, the area of erosions caused by regular I/R increased significantly above that recorded in those with vehicle pretreatment and this effect was accompanied by the significant fall in the GBF as compared to the respective value obtained in preconditioned rats. Pretreatment with Vioxx, a specific inhibitor of COX-2 or resveratrol, a specific inhibitor of COX-1, also resulted in a significant increase in the area of gastric lesions accompanied by a marked fall in the GBF but these alterations were significantly smaller than those attained with indomethacin (Figure 6).

The generation of PGE_2 in the intact gastric mucosa averaged 128±12 ng/g of wet tissue weight (Table 1). The exposure of gastric mucosa to regular I/R that caused gastric lesions produced a significant decrease (about 50%) in the PGE_2 generation as compared to the values recorded in the intact gastric mucosa (68±8 ng/g vs 128±12 ng/g of wet tissue weight, Table 1). The administration of indomethacin (5 mg/kg i.p.) that suppressed mucosal generation of PGE_2 by about 90% increased significantly the mean area of I/R lesions and this effect was accompanied by a significant fall in the GBF (Figure 6, Table 1). Vioxx failed to affect significantly the generation of PGE_2 in the gastric mucosa not exposed to I/R. When Vioxx was applied i.g. in a dose of 5 mg/kg, the significant increase in gastric lesions and the fall in the GBF and PGE_2 were observed. Resveratrol, which also augmented significantly the area of gastric lesions induced by I/R produced a significant fall in the PGE_2 generation (Table 1).

As shown in Figure 6, the area gastric lesions measured after I/R preceded by standard preconditioning was significantly higher and the GBF was significantly lower in this series of experiments in rats pretreated with indomethacin (5 mg/kg i.p.), Vioxx (5 mg/kg i.g.) and resveratrol (10 mg/kg i.g.) applied 30 min before short ischemia as compared to that recorded in vehicle-treated animals. Addition of PGE_2 (1 μg/kg i.g.), which by itself failed to influence significantly the I/R lesions (data not shown), attenuated significantly the enhancement in area of these lesions and accompanying fall in GBF induced by indomethacin (Figure 6). PGE_2 added to Vioxx or resveratrol abolished completely the increase in area of gastric lesions and accompanying fall in the GBF induced by administration of these COX-inhibitors (Figure 6).

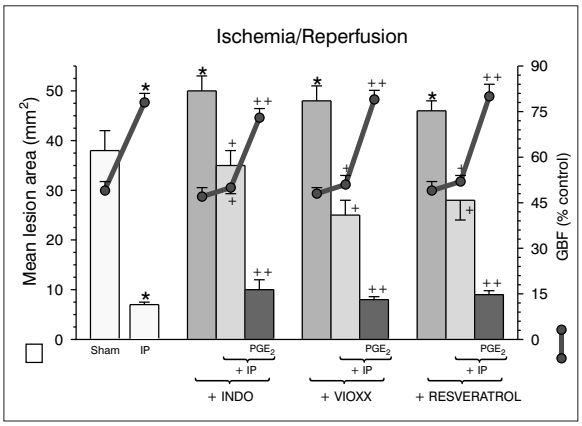

Figure 6. Effect of standard IP with or without pretreatment with indomethacin (5 mg/kg i.p.), Vioxx (5 mg/kg i.g.) and resveratrol (10 mg/kg i.g.) administered with or without addition of 16, 16 dm prostaglandin E_2 (PGE_2 1 μg/kg i.g.) on the area of gastric lesions and accompanying changes in the GBF induced by the exposure to regular 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 treatment with COX inhibitors. Double cross indicates a significant change as compared to the value obtained in rats without PGE_2 administration.

Table 1. Effect of standard ischemic preconditioning (IP) without or with the pretreatment with resveratrol (10 mg/kg i.g), Vioxx (5 mg/kg i.g.) and indomethacin (5 mg/kg i.p.), on the mucosal generation of PGE_2 in gastric mucosa exposed to regular ischemia/reperfusion. 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 change as compared to the respective value obtained in gastric mucosa not exposed to

Type of test	PGE_2 generation (ng/g)
Intact	128 ±12
Without ischemia/reperfusion:	
IP	154 ± 10*
Indomethacin	33 ± 4*
Vioxx	118 ± 13
Resveratrol	104 ± 9*
With ischemia/reperfusion:	
Sham	68 ± 8*
IP	118 ± 6+
Indomethacin + IP	18 ± 3++
Vioxx + IP	64 ±5++
Resveratrol + IP	55 ± 4++

Effect of L-NNA on gastric lesions, gastric blood flow and NO production in gastric mucosa exposed to I/R with or without gastric preconditioning

Figure 7 shows the results of tests with standard preconditioning with or without addition of L--NNA or the combination of L-NNA plus L-arginine or D-arginine on the area of gastric luminal contents of NO_3^-/NO_2^- and the GBF. The pretreatment with short ischemia resulted in usual attenuation of lesion area and an increase in the GBF and produced a significant rise in luminal contents NO_3^-/NO_2^-. L-NNA applied i.g. in a dose of 20 mg/kg, aggravated significantly the lesions induced I/R and decreased the GBF and luminal release of NO degradation products as compared to those in vehicle-treated animals. Such treatment with L-NNA abolished the decrease in I/R lesions, the rise in the GBF and the production of NO into gastric lumen recorded in animals subjected to gastric preconditioning applied before I/R (Figure 7). Addition of L-arginine but not D-arginine to the combination of L-NNA and short ischemic restored the protective effect, the rise in GBF and luminal NO_3^-/NO_2^- content to the levels observed in rats pretreated with short ischemic (Figure 7).

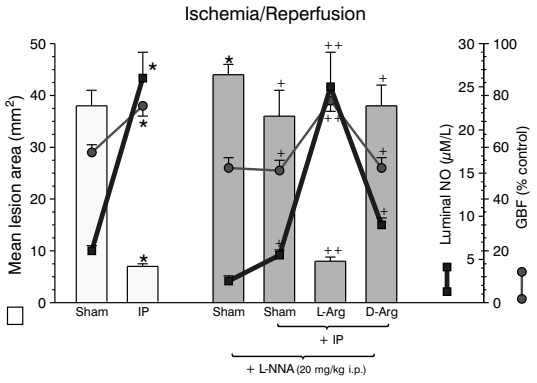

Figure 7. Effect of standard IP with or without pretreatment with N^G-nitro-L-arginine (L-NNA 20 mg/kg i.p.) applied with or without the combination with L-arginine (L-Arg, 200 mg/kg i.g.) or D-arginine (D-Arg, 200 mg/kg i.g.) on the area of gastric lesions and accompanying changes in the GBF and gastric luminal NO concentration induced by the exposure to regular 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 treatment with L-NNA. Double cross indicates a significant change as compared to the value obtained in rats without L-Arg administration.

Effect of exogenous adenosine and suppression of adenosine receptors by 8-phenyl theophylline on the gastric lesions induced by I/R with or without short ischemic

Pretreatment with adenosine (10 mg/kg i.g.) attenuated significantly the lesions induced by regular I/R and increased the GBF with the extent similar to that observed with standard preconditioning (Figure 8). An non-selective antagonist of adenosine receptors, 8-p-sulphophenyl theophylline (SPT, 10 mg/kg i.g.), which by itself failed to influence the area of gastric lesions and accompanying increase in the GBF, reduced significantly the protection and rise in the GBF caused by both, gastric preconditioning or pretreatment with exogenous adenosine against lesions induced by I/R (Figure 8).

Expression of COX-1 and COX-2 mRNA by RT--PCR in gastric mucosa exposed to I/R lesions with or without standard preconditioning

Figure 9 (right panel) shows expression of β-actin, COX-1 and COX-2 mRNA in the gastric mucosa of intact rats treated with vehicle and not exposed to regular I/R or those exposed to I/R with or without standard ischemic preconditioning and killed immediately after the end of regular I/R. The expression of β-actin mRNA was well-preserved in the mucosal samples taken both from rats treated with vehicle (control) or exposed to I/R and tested at various time intervals (Figure 9). The COX-1 mRNA was detectable in the vehicle-treated gastric mucosa as well as in the mucosa exposed to regular I/R at all time intervals after the end of I/R. Ratio mRNA COX-1 over β-actin revealed that the expression of COX-1 mRNA was similar in gastric mucosa exposed to I/R with or without I/R (Figure 9, left panel). In contrast, the signal for COX-2 mRNA was not detected in vehicle control animals but has been traced in rats exposed to I/R and in those treated with short ischaemia prior to exposure to I/R (Figure 9, right panel). The ratio of COX-2 mRNA over β-actin mRNA showed the expression of COX-2 in preconditioned mucosa was significantly higher than that recorded in animals immediately after the end of I/R without gastric preconditioning (Figure 9, left panel).

DISCUSSION

This study demonstrates that preconditioning of the gastric mucosa with short episodes of ischemia in the stomach exerts significant protection against

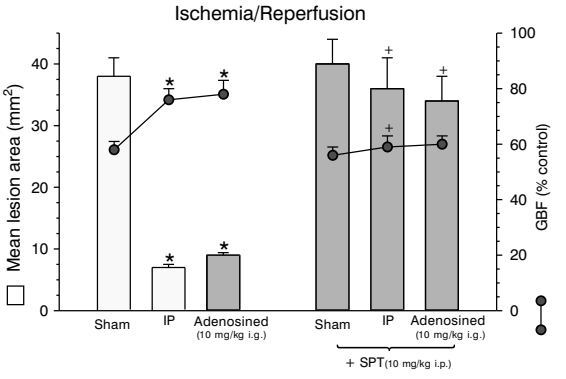

Figure 8. Effect of standard IP and adenosine (10 mg/kg i.g.) applied alone or combined with 8-p-sulphophenyl theophylline (SPT; 10 mg/kg i.p.) on gastric lesions and accompanying changes in the GBF induced by the exposure to regular I/R. Results are mean±SEM of 6–8 rats. Asterisk indicates a significant 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 treatment with SPT.

lesions caused by longer exposure to regular I/R (I/R) and this protective effect depends upon the time of ischemic episodes. To our best knowledge, this is the first demonstration that phenomenon of preconditioning described originally in various organs including heart, lungs, liver and intestine [1–6,9,10], occurs also in the stomach resulting in the limitation of the mucosal damage evoked by the I/R. Our study indicates that gastric preconditioning may represent one of the most powerful protective intervention against the damage induced by severe I/R as well as various necrotizing substances including 100 % ethanol in the stomach.

Based on our results, it is reasonable to assume that gastric preconditioning involves several mediators including prostaglandin derived from COX-1 and COX-2 activity, NO and adenosine that appear to play a key role in the mechanism of this protection probably by causing vasodilatation and enhancement the GBF. This notion is supported by the fact that protection and accompanying rise in the GBF induced by gastric preconditioning were significantly attenuated by inhibition of COX-1 and COX-2 and by L-NNA suppressing NO-synthase activity. The involvement of the above mediators is supported by the effect of concurrent treatment with synthetic PGE_2 to compensate for the deficiency of endogenous prostaglandin and by L-arginine to provide a substrate for NO synthase. Furthermore, we found that the protective and hyperemic effects of preconditioning against I/R were antagonized by SPT, an antagonist of adenosine receptors and that exogenous adenosine attenuated significantly gastric lesions induced by I/R with the extent similar to that observed after standard ischemic preconditioning suggesting that adenosine may be the major factor contributing to the beneficial effect of gastric preconditioning in the stomach.

Previous studies demonstrated that prostaglandins applied exogenously or generated endogenously in the gastric mucosa, exhibit high activity in preventing the mucosal damage induced by necrotizing substances including boiling water [13,14]. Adaptive cytoprotection was introduced originally by Robert and his associates [13] to describe the protective activity of endogenous prostaglandin generated within gastric mucosa by mild topical irritants such as 20% ethanol or 5% NaCl to against severe mucosal damage induced by strong irritants such as 100% ethanol or 25% NaCl. We demonstrated previously [14] 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 by 25% NaCl.

Besides prostaglandin also NO was later on implicated as mediator of this adaptation [15–17] and in fact some reports suggested that prostaglandin may not be primary mediators of this mucosal adaptive protection [37]. It is of interest that this protective mucosal mild-irritation was proposed to act locally because mild irritants failed to exhibit any protective activity when applied systemically [14].

Preconditioning refers to a phenomenon in which a tissue is rendered resistant to the deleterious effects of severe and prolonged ischemia followed by reperfusion by previous exposures to brief periods of vascular occlusion [4]. While the beneficial effects of preconditioning were first demonstrated in the myocardium [2], it is now evident that this preconditioning protects against postischemic damage of brain, kidney, skeletal muscle and gastrointestinal organs including small bowel and liver [1–3,6,11,12]. Mechanism of protection induced by preconditioning has not been fully explained but activation of adenosine A1 receptors and ATP-sensitive potassium channels in the heart as well as an inhibition of neutrophil activation and emigration in the intestine were implicated in this phenomenon [4]. No attempts were made however, to examine whether preconditioning in the stomach

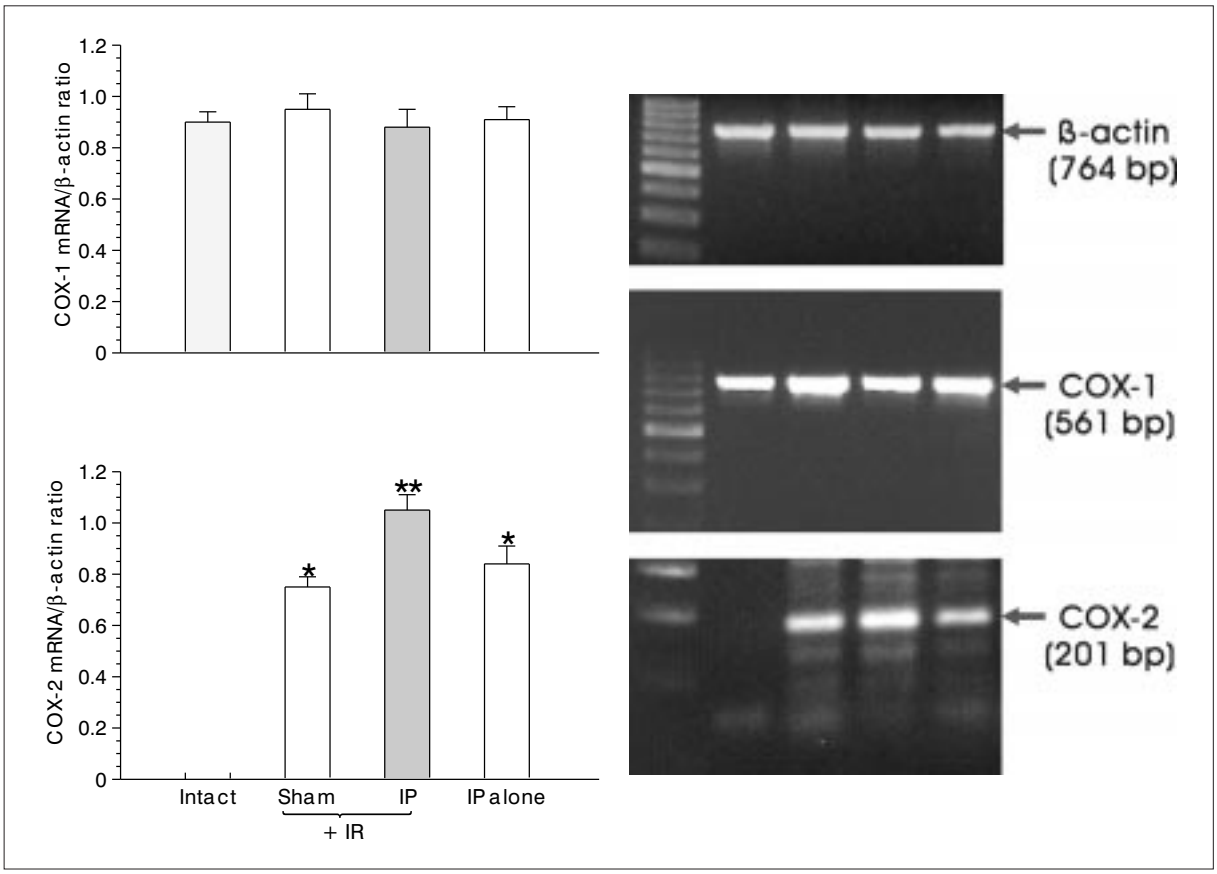

Figure 9. Messenger RNA expression for β-actin, cyclooxygenase (COX)-1 and cyclooxygenase (COX)-2 mRNA (right panel) and assessment of mucosal gene expression for COX-1 and COX-2 by the intensity of COX-1, COX-2 mRNA/β-actin mRNA ratio in intact gastric mucosa (lane 1), sham plus regular I/R (lane 2), standard IP plus regular I/R (lane 3) and IP alone (lane 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. Cross indicates a significant change as compared with the value obtained in sham- and IP alone treated animals.

could enhance its mucosal resistance against the damage induced by subsequent exposure to prolonged and severe I/R.

In present study we compared the effect of preconditioning on the gastric mucosa injured by I/R with that exhibited by mild irritants. We confirmed that gastric mucosa pretreated with mild irritants such as 5% NaCl, 20% ethanol or 5 mM taurocholate, acquires a tolerance against subsequent damaging insults of strong irritants such as 25% NaCl, 100% ethanol of 80 mM taurocholate and we found for the first time that this increased tolerance was also achieved by gastric preconditioning. Furthermore, we have demonstrated for the first time that the preconditioning protected gastric mucosa also against lesions induced by strong irritants such as 100% ethanol, 25% NaCl and 80 mM taurocholate indicating that the preconditioning like mild irritants [13,14] affords cross-protection against lesions caused by these strong irritants.

We attempted in this study to determine the possible mechanism of gastric preconditioning and the role of endogenous prostaglandin, NO and adenosine as potential mediators of this protection in the stomach. Numerous studies have documented that prostaglandin derived from the activity of the COX isoforms, especially COX-1, play an important role in mechanism of gastric integrity, gastroprotection [13,14] and ulcer healing [26]. Recently prostaglandin-derived from cyclooxygenase-2 were implicated in the protective and ulcer healing activities of growth factors by the demonstration that COX-2 is upregulated in the edge of gastric ulcer and that this is significantly enhanced by the treatment with growth factors [38]. Moreover, endogenous prostaglandin derived from COX-1 and COX-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 COX-2 mRNA [23]. The involvement of these

arachidonate products in the mechanism of preconditioning has not been fully elucidated but it was suggested that in the heart certain prostaglandin such as prostacyclin, that is released from ischemic myocardium, may limit the extent of heart infarct and attenuate ventricular arrhythmia and that inhibition of COX 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 COX-2 is overexpressed in the preconditioned gastric mucosa, at the levels of both, mRNA and protein, while COX-1 mRNA remains unchanged. Moreover, the suppression of the PG biosynthesis by non-selective (indomethacin) and selective COX-1 (resveratrol) or COX-2 (Vioxx) inhibitors attenuated or abolished the protective and hyperemic effects of gastric preconditioning. Furthermore minute amounts of synthetic PGE_2 analog added to these COX inhibitors restored the protection by preconditioning reinforcing the notion that endogenous PG produced in excessive amounts by COX-2, play an important role in the mechanism of gastric preconditioning.

Previous studies revealed that NO released from vascular endothelium, sensory afferent nerves or gastric epithelium is essential for the gastroprotection and ulcer healing [27–30,39,40]. 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 [40]. Our present study implies that NO could also participate as a candidate mediator in gastric preconditioning.

In agreement to previous findings that NO derived from L-arginine metabolic pathway could contribute to the mechanism of preconditioning in the liver injury induced by hepatic I/R [5,41] we found that the gastroprotection afforded by gastric preconditioning is accompanied by the rise in the gastric blood flow probably due to enhanced production of NO in the gastric mucosa. Both these effects occurring after preconditioning were completely abolished in rats with suppressed NO synthase activity by L-NNA. 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.

We also tested the hypothesis, suggested by others in hepatic preconditioning [6], that adenosine plays a crucial role in the mechanism of gastric preconditioning. Indeed, the beneficial effects of preconditioning were blocked, at least in part by the administration of non-selective adenosine receptor antagonist SPT. Furthermore the pretreatment with adenosine in non-preconditioned animals resembled the protective effect of preconditioning against the I/R gastric injury. These observations are consistent with the hypothesis that locally released adenosine during preconditioning could trigger the preconditioning phenomenon via activation of adenosine receptors.

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