Importance of nitric oxide (NO) and adenosine in the mechanism of gastric preconditioning induced by short ischemia

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Abstract

Purpose: Gastric mucosa subjected to repeated brief episodes of ischemia exhibits an increased resistance to damage caused by a subsequent prolonged ischemic insult and this is called gastric preconditioning. In this study, L-NNA, a non-selective NO-synthase inhibitor, and aminoguanidine, a relative inhibitor of inducible NO-synthase (iNOS), were applied prior to short ischemia (occlusion of celiac artery 1-5 times for 5 min) followed by a subsequent exposure to 0.5 h of ischemia and 3 h of reperfusion (I/R).

Material and methods: Male Wistar rats were used in all studies.

Results: Short ischemia reduced significantly I/R-induced lesions while raising significantly the GBF and luminal NO content. These effects were attenuated by L-NNA and aminoguanidine and restored by addition of L-arginine and SNAP to L-NNA and aminoguanidine. Pretreatment of with adenosine (10 mg/kg i.p.) significantly reduced I/R lesions and accompanying fall in the GBF induced by I/R. These protective and hyperemic effects of standard preconditioning and adenosine were significantly attenuated by pretreatment with 8-phenyl theophylline (SPT, 10 mg/kg i.g.), an antagonist of adenosine A_1 and A_2 receptors.

Conclusions: We conclude that gastric ischemic preconditioning is considered as one of the major protective mechanism in the stomach that involves key vasodilatory mediators such as NO and adenosine.

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Introduction

Preconditioning to ischemic tolerance is a phenomenon in which brief episodes of a subtoxic insult induce a robust protection against deleterious effect of subsequent, prolonged, lethal ischemia [1]. These protective effects of ischemia preconditioning were first described in the heart by Murry and coworkers [2]. Since that time, preconditioning has been shown to reduce the extent of myocardial infarct size as well as the damage of skeletal muscle, brain, kidney and intestine induced by subsequent exposure to prolonged severe ischemia/reperfusion in a variety of species [3-6] but the mechanism of this protection remains unknown.

Cardioprotective effect of ischemia preconditioning is well documented because repeated short episodes of ischemia due to the temporal occlusion of coronary vessels were shown to prevent lethal cell injury of the myocardium against the damage induced by long-term severe ischemia/reperfusion [7]. In another report, ischemic preconditioning of rat mesenteric venules led to enhance bioavailability of nitric oxide (NO) and abolished oxidant production as well as attenuated the leukocyte adhesion and emigration in the mesentery [8]. This was the first evidence that ischemia preconditioning can also exists in the gut, at least in part, preventing the mesenteric microvascular barrier dysfunction and inactivation of NO in the intestine. Recently, we have shown that brief episodes of ischemia of the stomach afforded protection against the gastric mucosal damage induced by prolonged ischemia/reperfusion insult by multifactorial mechanism involving endogenous prostaglandins, NO, adenosine and neuropeptides released from sensory afferent neurons [9]. This protection by ischemic preconditioning of gastric mucosa mimicked that exerted by

certain mild irritants such 20% ethanol, 5% NaCl or 5 mM taurocholate against the damage induced by these agents applied intragastrically in much larger necrotizing concentrations and called adaptive cytoprotection [10-12]. The protective action of mild irritants has been attributed to the activity of endogenous PG but besides PG the importance of other protective factors such as NO, nonprotein sulfhydryl compounds and sensory nerves were implicated in this phenomenon [13-17].

The mechanism of ischemic preconditioning remains unclear but adenosine, which is produced during the ischemic preconditioning was proposed to act as an initiator of this preconditioning in different organs such as heart and liver. Adenosine was effective in attenuating the injury caused by severe ischemia/reperfusion and this protection induced by ischemic preconditioning can be reversed by adenosine receptor antagonists [3,18]. The question remains whether adenosine mimics the protective effect of ischemia preconditioning in the stomach and can exert protection against gastric lesions caused by prolonged ischemia/reperfusion and if so which adenosine receptors are involved in this response of preconditioning.

This study was designed to determine the effect of the ischemia preconditioning in the stomach on gastric lesions induced by ischemia/reperfusion (I/R) and to elucidate the role of GBF, NO release into the gastric lumen and adenosine in the gastric mucosa subjected to ischemia 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 erosions were produced in 120 rats by the method originally proposed by [19]. 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. We attempted to determine 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. The second goal was to test 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 addition, short ischemia (occlusion of celiac artery for 5 min 1-5 times – ischemia preconditioning) was applied 30 min before subsequent exposure to longer 30 min of ischemia (also induced by clamping of celiac artery) followed by 3 h of reperfusion (I/R). Involvement of NO in the protective effect of gastric preconditioning.

The implication of NO in the effect of gastric preconditioning on damage induced by ischemia/reperfusion was determined by three ways: 1) by the use of N^G-nitro-L-arginine (L-NNA) applied i.p. in a dose of 20 mg/kg to suppress nonspecifically the activity of NOS [21] and aminoguanidine (10 mg/kg i.p.), a relative specific inhibitor of iNOS [21] to inhibit iNOS activity; 2) by the indirect measurement of NOS product i.e. NO in gastric lumen [22]; and 3) by addition to L-NNA of L-arginine, a substrate for NOS or D-arginine, which is not a substrate for NO [23]. The rats with gastric lesions induced by regular ischemia/reperfusion 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.) and aminoguanidine (10 mg/kg i.p.) 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; 4) D-arginine (200 mg/kg i.g.) plus L-NNA (20 mg/kg i.g.) combined with the preconditioning, and finally; 5) SNAP (5 mg/kg i.g.) plus aminoguanidine (10 mg/kg i.p.) 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 [24]. This method is based on the Griess reaction and generation of chromophore absorbing at 595 nm, according to the original procedure reported previously [25]. Since NO released by epithelial cells into the gastric lumen is quickly transformed into NO_3^- and NO_2^- [22], the sum of these products of NOS gives an index of production of NO by the enzyme in the gastric mucosa. In order to determine NOx, 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 and aminoguanidine with or without SNAP, the GBF was measured in the oxyntic mucosa in each group of animals in similar manner as described below 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 [26], 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 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 GBF was measured in the oxyntic gland area of the stomach by means of local H_2-gas clearance method using an electrolytic regional blood flow meter (Biomedical Science, Model RBF-2, Japan) as described previously [27]. The measurements were calculated in three areas of the mucosa and the mean absolute values

Figure 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 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.

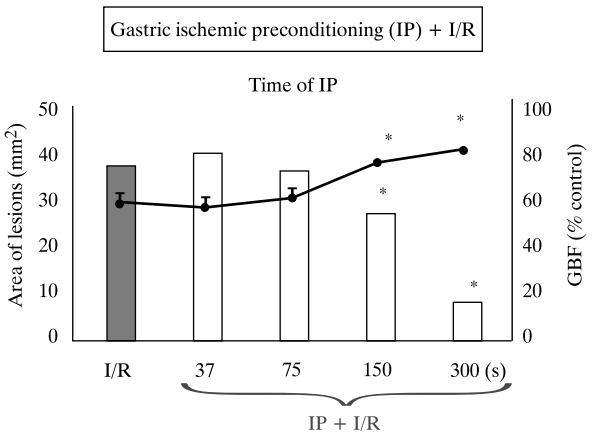
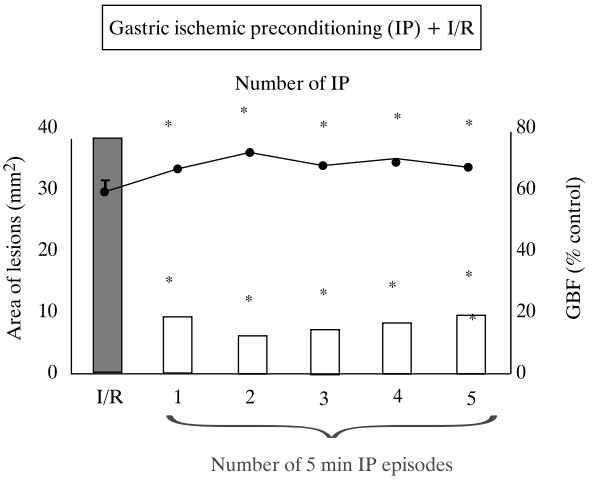


Figure 2. Mean area of gastric lesions (columns) and gastric blood flow (GBF) (lines) in the gastric mucosa of rats pretreated with sham (control) and various numbers of 5 min ischemic episodes. Results are mean ± SEM of 6-8 rats. Asterisk indicates a significant change as compared with the value obtained in sham-control animals.



(ml/100 g-min) of these measurements were calculated and expressed as percent changes from those recorded in control animals treated with vehicle.

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.

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

In order to select how many episodes of short ischemia is necessary to afford the maximal protective action against the damage evoked by prolonged I/R, we tested 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 ischemia/reperfusion. *Fig. 2* shows that 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 (2x5 min occlusion) did not result in any further significant reduction in lesion area caused by regular ischemia/reperfusion. 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 (*Fig. 2*). A single standard ischemic episode increased significantly the gastric blood flow by about 25% as compared to that recorded in sham-operated controls exposed to regular ischemia/reperfusion. 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 (*Fig. 2*).

Effect of L-NNA and aminoguanidine on gastric lesions, gastric blood flow and NO production in gastric mucosa exposed to ischemia/reperfusion with or without gastric preconditioning. *Fig. 3* 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 GBF. The pretreatment with short ischemia resulted in usual attenuation of lesion area and an increase in GBF and produced a significant rise in luminal contents NO_3^-/NO_2^-. L-NNA applied i.p. in a dose of 20 mg/kg, aggravated significantly the lesions induced ischemia/reperfusion and decreased the gastric blood flow and luminal release of NO degradation products as compared to those in vehicle-treated animals. Such treatment with L-NNA abolished the decrease in ischemia/reperfusion lesions, the rise in gastric blood flow and the production of NOx into gastric lumen recorded in animals subjected to gastric preconditioning applied before I/R (*Fig. 3*). Addition of L-arginine but not D-arginine to the combination of L-NNA and short ischemic restored the protective effect, the rise in gastric blood flow and luminal NO_3^-/NO_2^- content to the levels observed in rats pretreated with short ischemia (*Fig. 3*). As shown in *Tab. 1*, pretreatment with aminoguanidine by itself failed to affect gastric

Figure 3. Effect of standard ischemic preconditioning (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 (columns) and accompanying changes in the gastric luminal NO concentration (lines) induced by the exposure to regular ischemia/reperfusion (I/R). Results are mean ± SEM of 6-8 rats. Asterisk indicates a significant change as compared with the value obtained in sham-control gastric mucosa. Cross indicates a significant change as compared with the value obtained in rats without treatment with L-NNA. Double cross indicates a significant change as compared to the value obtained in rats without L-Arg administration.

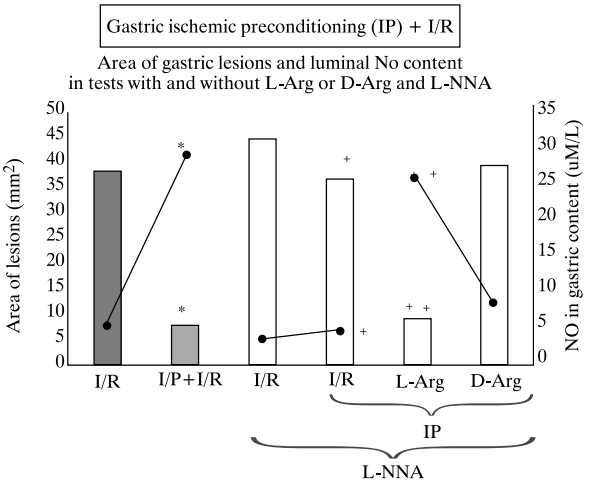
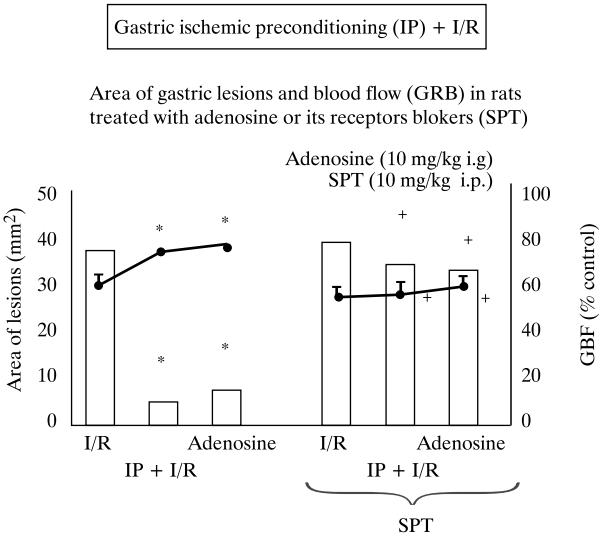


Figure 4. Effect of standard ischemic preconditioning (IP) and adenosine (10 mg/kg i.g.) applied alone or combined with 8-p-sulphophenyl theophylline (SPT; 10 mg/kg i.p.) on the area of gastric lesions (columns) and accompanying changes in the GBF (lines) induced by the exposure to standard ischemia/reperfusion. 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 induced by I/R but significantly attenuated the decrease in the area of I/R-induced gastric lesions and accompanying rise in the GBF caused by gastric preconditioning. Addition of SNAP, a NO-donor to aminoguanidine, restored the protective and hyperemic effects of short ischemia against prolonged I/R (*Tab. 1*).

Effect of exogenous adenosine and suppression of adenosine receptors by 8-phenyl theophylline on the gastric lesions induced by ischemia/reperfusion with or without short ischemia. Pretreatment with adenosine (10 mg/kg i.g.) attenuated significantly the lesions induced by regular ischemia/reperfusion and increased the gastric blood flow with the extent similar to that observed with standard preconditioning (*Fig. 4*). A non-selective antagonist of adenosine receptors, 8-p-sulphophenyl theophylline (10 mg/kg i.g.), which by itself failed to influence the area of gastric lesions and accompanying increase in the gastric blood flow, reduced significantly the protection and rise in the gastric blood flow caused by both, gastric preconditioning or pretreatment with exogenous adenosine against lesions induced by ischemia/reperfusion (*Fig. 4*).

Discussion

This study shows that the preconditioning of the gastric mucosa with short episodes of ischemia in the stomach exerts significant protection against lesions caused by longer exposure to regular I/R and documented that this protection may involve NO. Furthermore, we found that the protective and hyperemic effects of preconditioning against ischemia/reperfusion were antagonized by 8-p-sulphophenyl theophylline (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 also contribute to the beneficial effect of gastric preconditioning in the stomach.

The phenomenon of preconditioning was described originally in various organs including heart, lungs, liver and intestine [1,2,4-6,8,28], resulting in the limitation of the mucosal damage evoked by the I/R. We demonstrated recently that gastric preconditioning may represent an important gastroprotective mechanism against the damage induced by severe I/R as well as it can exhibit protective action against the damage caused by various necrotizing substances including 100% ethanol, 25% NaCl and 80 mM taurocholate in the stomach [9].

In this study, we attempted to determine the possible mechanism of gastric preconditioning with the major focus on the role of endogenous NO and adenosine as potential mediators of this protection in the rat stomach. Previous studies revealed that NO released from vascular endothelium sensory afferent nerves or gastric epithelium is essential for the gastroprotection and ulcer healing [17,20,22,23,27,29]. Furthermore, non-steroidal anti-inflammatory drugs (NSAIDs) that contain NO moiety are believed to counteract the vasoconstriction effects of conventional NSAIDs therefore limiting the gastrotoxicity and ulcerogenic effects of NSAID-therapy [30]. We have shown previously that administration of suppression of NO-synthase activity with NO-inhibitors abolished the gastroprotective activity of capsaicin in the stomach and delayed healing of chronic gastric ulcers [17] but the question which enzyme

Table 1. Effect of standard ischemic preconditioning (IP) without or with the pretreatment with aminoguanidine or SNAP (5 mg/kg i.g) added to aminoguanidine on the area of gastric lesions induced by ischemia/reperfusion (I/R) and the changes in the GBF. Results are mean ± SEM of 8-10 rats. Asterisk indicates a significant change as compared to the value obtained in vehicle-treated gastric mucosa. Cross indicates a significant change as compared to the value obtained in gastric mucosa exposed to I/R. Double cross indicates a significant change as compared to the respective value obtained in gastric mucosa pretreated with aminoguanidine.

Type of test	Mean lesion area (mm^2)	GBF (% Control)
I/R	44 ± 3	49 ± 4
IP + I/R	6 ± 0,4*	72 ± 5*
Aminoguanidine +I/R	48 ± 5	47 ± 3
Aminoguanidine +IP + I/R	32 ± 6+	63 ± 6+
SNAP + aminoguanidine + IP + I/R	10 ± 2,5++	68 ± 5++

becomes a source of NO in the protective response of transient short ischemia against I/R-induced gastric lesions has not been fully clarified.

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 [28]. Previous studies revealed that the mechanism of gastric preconditioning is multifactorial in nature and may depend upon several mediators including prostaglandin derived from cyclooxygenase-1 and cyclooxygenase-2 activity and CGRP released from sensory afferent nerve endings [9,31,32]. These mediators play a key role in the mechanism of this protection possibly by causing vasodilatation and enhancing the gastric blood flow. Our present report is in keeping with this hypothesis because the protection and accompanying rise in the gastric blood flow induced by gastric preconditioning were significantly attenuated by non-selective suppression of NO-synthase activity by L-NNA and by relative inhibitor of iNOS, aminoguanidine. The involvement of NO is supported by the fact that the concurrent treatment with L-arginine to provide a substrate for NO synthase and with SNAP, a potent donor of NO, added to L-NNA and aminoguanidine, respectively, restored the protective and hyperemic activity of gastric preconditioning against I/R injury. It is known that NO and various vasodilatatory neuropeptides such as CGRP are released from sensory nerves and functional ablation of sensory nerves with capsaicin was shown to attenuate both, protection and gastric hyperemia induced by gastric preconditioning indicating that NO derived from afferent nerves could be also considered as an important mediator of protection induced by short ischemia [32].

We also tested in this report the hypothesis, suggested by others in hepatic preconditioning [18], 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 might trigger the gastroprotection afforded by preconditioning via activation of adenosine receptors.

As mentioned, the ischemic preconditioning has been best studied in coronary artery disease, where transient ischemia was found to reduce the degree of infarction following severe and sustained ischemia. Stefano et al. [33] postulated that short ischemia mimics the cell response to normal dips in ATP levels caused by metabolic demands; the action involving NO originating from cNOS and resulting in temporary down-regulation of the cell excitatory state. This suggests that the major enzyme involved in the short-term gastric preconditioning appears to be cNOS rather than iNOS. This notion is in keeping with the original findings in heart that ischemic preconditioning elicited a biphasic response in cardiac NOS activity induced by transient and late preconditioning, namely, an immediate activation of cNOS and late upregulation of iNOS. This upregulation of cNOS together with downregulation of transcription factor, NFkB, were postulated to play a major role in the short ischemia-induced protection against the damage caused by the next severe insult. We did not look in this study which NOS isofrom is involved in gastric preconditioning but it is rationale to consider a distinctive role of NOS isoforms in gastric preconditioning, with cNOS serving as the trigger of protection by transient ischemia preconditioning and with the iNOS as the possible mediator of late preconditioning.

In summary, 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 and activation of adenosine receptors in the gastric mucosa. Both these effects occurring after preconditioning were significantly attenuted in rats with suppressed NO synthase activity by L-NNA and in those with the blockade of adenosine receptors by SPT. 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. Similar reversal of protective effects of gastric preconditioning against I/R was observed in rats with capsaicin-induced deactivation of sensory nerves [32] suggesting that NO, adenosine and sensory nerves cooperate in the beneficial effects of short ischemic episodes against severe lesions caused by prolonged ischemia/reperfusion.

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