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Neuronal Nitric Oxide Mediates the Anti-Inflammatory Effects of Intestinal Ischemic Preconditioning

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ABSTRACT

Background: Ischemic preconditioning (IPC) can provide a defense against ischemia–reperfusion (IR)-induced acute inflammation and barrier dysfunction in many organs. Because nitric oxide (NO) has been implicated as a trigger or mediator in the IPC mechanism and because neuronal NO synthase (nNOS) is a dominant isoform of NOS in the gastrointestinal tract, our aim was to investigate the role of nNOS in IPC-induced protection after mesenteric IR.

Materials and methods: Intestinal IR was induced in sodium pentobarbital–anesthetized dogs by clamping the superior mesenteric artery for 60 min followed by 2 h of reperfusion (IR group; $n = 7$). In further groups, IPC was used (three cycles of 5-min ischemia/5-min reperfusion periods) before IR in the presence or absence of selective inhibition of nNOS with 7-nitroindazole (5 mg/kg, intravenously, in a bolus 15 min before IPC, $n = 6$ each). Changes in mesenteric vascular resistance, intramucosal pH (pHi), and small bowel motility were monitored. Plasma nitrite/nitrate levels, intestinal NO synthase activity, leukocyte accumulation, mast cell degranulation, and histologic injury were also determined.

Results: Ischemia significantly decreased mesenteric vascular resistance and pHi, whereas IR induced a temporary bowel hypermotility and acute inflammatory reaction. IPC facilitated pHi recovery, attenuated motility dysfunction, elevated NOS-dependent NO production, and reduced leukocyte accumulation, mast cell degranulation, and mucosal injury. Pretreatment with 7-nitroindazole halted the IPC-induced increase in NO availability, pHi recovery, and the anti-inflammatory and morphologic effects.

Conclusions: Our data demonstrate that NO generated by intestinal nNOS plays a pivotal role in IPC-linked tissue protection by inhibiting an IR-related acute inflammatory response.

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Introduction

In past decades, the protective potential of ischemic preconditioning (IPC) has been demonstrated in a number of organs, including the gastrointestinal tract.^{1–3} The attenuating effect of IPC against an ischemia–reperfusion (IR)-induced inflammatory response in the intestine⁴ involves reduced activation of transcription factor nuclear factor-kappa binding⁵ and cytoprotection through inhibition of apoptosis.⁶ Preservation of enteric nerve function and prevention of intestinal motility dysfunction represent another manifest protective effect of IPC, at least in rats.⁷

Among many possible triggers and mediators, nitric oxide (NO) seems to play a key initiating role in IPC-linked protection. This hypothesis is supported by the immediate increase in tissue nitrite and nitrate (NO_x) levels after IPC in the rat intestine.² It has also been shown that endogenous NO production by NO synthases (NOSs) plays a regulatory role in intestinal motility after reperfusion⁸ and the preservation of mucosal barrier function on IR injury.⁹ Furthermore, NO-releasing compounds or NO donors (e.g., SIN-1, sodium nitroprusside, CAS 754, and FK409) exerted similar protection against mucosal injury in rats,¹⁰ cats,¹¹ and dogs¹² as well.

Among the two calcium-dependent constitutive isoforms, neuronal NO synthase (nNOS or type 1 NOS) is a dominant isoenzyme in the small intestine of both rodents¹⁰ and large animals.¹³ Nevertheless, the role of nNOS in intestinal IR injury–induced acute inflammatory reactions is still debated. A number of studies have demonstrated a proinflammatory role for nNOS,^{14–16} but anti-inflammatory properties have also been described.¹⁷ Furthermore, treatment with nNOS inhibitor 7-nitroindazole (7-NI) in healthy rats for 4 d exhibited higher intestinal myeloperoxidase (MPO), inducible NOS, and nuclear factor-kappa binding activity with concomitant lower IkB α expression.¹⁸ The paradoxical role of nNOS-mediated NO signaling during inflammation may be due to the differences in the severity and time course of model diseases (i.e., colitis, sepsis, or cerebrovascular inflammation) and species differences (i.e., rodents versus larger animals) as well.

In the present study, we aimed to investigate the possible role of nNOS in the local IPC-induced protection in the gastrointestinal tract of a large animal model, where innervation is similar to that of humans.¹⁹ To this end, 7-NI, a specific nNOS inhibitor with a high neuronal uptake,²⁰ was administered before IPC in anesthetized dogs. Particular emphasis was placed on intestinal motility function and inflammatory reactions in the early reperfusion phase when the parallel involvement of NOS activation was also determined.

Materials and methods

The experiments complied with the ARRIVE guidelines and the project were approved by the National Scientific Ethics Committee on Animal Experimentation (National Competent Authority) in Hungary under license number V./148/2013. The study was performed in compliance with EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and the National Institutes of Health

guidelines on the use of experimental animals (NIH Publications No. 8023, revised 1978).

Surgical preparation

Outbred dogs of either sex with average body weight of 12.7 ± 2 kg were obtained from the Animal House of the University of Szeged ($n = 19$). The animals were anesthetized intravenously with sodium pentobarbital (30 mg/kg intravenously), and polyethylene catheters were surgically inserted into the femoral artery and vein to measure blood pressure and administer fluids or drugs, respectively. All animals received a continuous infusion of Ringer's lactate at a rate of 10 mL/kg/h during the experiments. After median laparotomy, the root of the superior mesenteric artery (SMA) was dissected free, and an ultrasonic flow probe (Transonic Systems Inc, Ithaca, NY) was placed around the exposed SMA to measure mesenteric blood flow. A branch of a tributary of the ileal vein supplying the terminal part of the ileum was cannulated with a 2-F polyethylene catheter to measure mesenteric venous pressure so as to obtain blood samples (see later).

Experimental protocol

Ten male and nine female outbred dogs were randomly allocated to IR ($n = 7$; male = 4 and female = 3) and IPC ($n = 12$; male = 6 and female = 6) groups (Fig. 1). In the IR group, mesenteric ischemia was elicited by occluding the SMA for 60 min with a miniclip, which was followed by 2 h of reperfusion. In the IPC group, animals were assigned into 7-NI-treated or vehicle groups and were treated with the selective nNOS inhibitor 7-NI (5 mg/kg; $n = 6$; male = 3 and female = 3) or saline vehicle ($n = 6$; male = 3 and female = 3) in an intravenous bolus 15 min before IPC. IPC was induced in two groups by clamping the SMA using three cycles of 5-min ischemia/5-min reperfusion periods starting 60 min before IR. Stock solution of 7-NI (Sigma–Aldrich Co, St. Louis, MO) was dissolved in a mixture of 75% dimethyl sulfoxide/saline and stored at -20°C ; the aliquots were diluted in 3 mL of 0.9% saline before administration. In the IR group, animals were treated with saline vehicle at a matching time point.

Blood samples were collected from the mesenteric vein for later determination of plasma NO_x levels at different time points (Fig. 1). Tissue biopsies were also harvested from the intestine for further biochemical/histologic (e.g., MPO, NOS activity, and mast cell (MC) degranulation) analyses (at baseline as well as at the 15th and 120th min of reperfusion).

Hemodynamic and blood gas measurements; calculation of mucosal pH

The peripheral arterial and mesenteric venous pressures (using Statham P23 Db transducers) were registered with a computerized data acquisition system (Haemosys 1.17 Experimetria Ltd, Budapest, Hungary). Mesenteric vascular resistance was calculated with a standard formula. A silastic balloon catheter (TGS Tonomitor; Tonometrics Inc, Worcester, MA) was introduced through a small enterotomy into the intestinal lumen. Arterial blood gases and intramucosal pCO₂

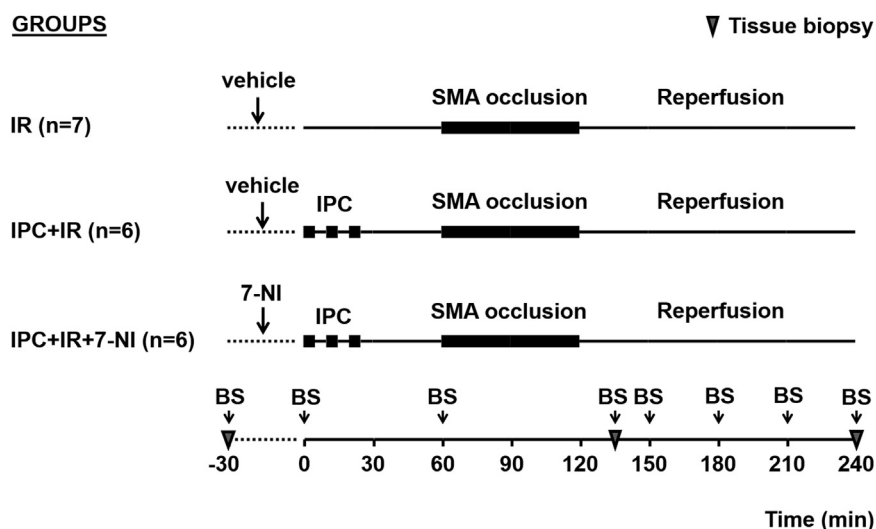


Fig. 1 – Experimental protocol. In the first group, 60 min of ischemia was induced by occluding the SMA, which was followed by 120 min of reperfusion (IR). In the two other groups, IR was preceded by IPC, which was elicited by inducing three cycles of 5-min ischemia/5-min reperfusion periods in the presence and absence of a bolus injection of 7-NI (5 mg/kg) 15 min before IPC (IPC + IR + 7-NI and IPC + IR groups, respectively). IR and IPC + IR groups were treated with a vehicle of 7-NI (a mixture of 75% DMSO/saline) at corresponding time points. As indicated, blood samples from the mesenteric vein (BS) and tissue biopsies from the affected bowel area (marked by an arrowhead) were also taken for later assessments of plasma nitrate/nitrite levels and NOS and MPO activities, as well as to assess MC degranulation and mucosal injury.

were measured with a blood gas analyzer (AVL, Graz, Austria). Intestinal pH (pHi) was calculated using the modified Henderson–Hasselbach formula with a correction factor for 30 min of equilibration.

Intestinal motility measurement

To monitor the small bowel motility, a modified strain gauge transducer technique was applied,¹³ the transducers being sutured to the circular muscle layer of a terminal ileal segment. Motility index was calculated to estimate the neurogenic integrity of the intestine.²¹

Plasma nitrate/nitrite level measurements

Plasma NO_x concentrations were determined from samples taken at baseline ($t = -30$ min), before ischemia as well as the 15th, 60th, and 120th min of reperfusion by means of the Griess reaction.¹³

Measurement of NOS enzyme activity

NOS activity was determined in intestinal tissue biopsies based on the enzymatic conversion of ³[H]-L-arginine to ³[H]-L-citrulline using the Lowry method; the Ca²⁺-dependent and Ca²⁺-independent NOS activities were assessed in the presence and absence of Ca-calmodulin, respectively.¹³

Tissue MPO activity measurements

MPO, a marker of neutrophil granulocyte infiltration, was measured from mucosal biopsies using a standard photometric method.²²

Determination of MC degranulation

Ileal biopsies were placed into an ice-cold Carnoy's fixative, embedded in paraffin, sectioned (6 μ m), and stained with Alcian blue and Safranin O. Histologic analysis was performed in coded sections by one investigator (S.V.) at 40 \times optical magnification in a blinded fashion. MCs were differentiated based on the location of granules, injury to the cell membrane, and volume of the cytoplasm. Intact cells were characterized by (1) intact membrane and (2) cytoplasmic granules, while criteria for degranulated cells were specified by (1) cell membrane injury, (2) reduced cytoplasmic volume and (3) loss of cytoplasmic granules. The percentage of degranulation (MC %) was calculated based on the number of intact MC (iMC) and degranulated MC (dMC) mast cells: MC% = dMC/(dMC + iMC).²³

Determination of morphologic changes in the structure of the small intestine

Analysis of morphologic injury to the same ileal biopsies was performed on slides stained with hematoxylin-eosin in coded slides in a blinded fashion. Measurements of the height of different portions of the mucosa were conducted with a computer-assisted image analysis system (IVM Pictron, Budapest, Hungary). The extent of mucosal damage was determined on the basis of (1) villus height—total mucosal height ratio (%) and (2) villus height—crypt height ratio (%); these were determined for each villus of the current slide (at 3–5 high power fields at 4 \times magnification). Villus tip injury was also assessed using a modified version of Chiu's method for each villus of the current slide (at 4–7 high power fields at 10 \times magnification).²⁴

Statistical analysis

Data analysis was performed using a statistical software package (SigmaStat 13.0 version for Windows, Jandel Scientific, Erkrath, Germany) with nonparametric methods. Normality was tested with the Shapiro–Wilk test. Friedman's repeated measures analysis of variance on ranks was applied within the groups. Time-dependent differences from the baseline were assessed with Dunn's method. Differences between groups were analyzed with the Kruskal–Wallis (one-way analysis of variance on ranks) test, followed by Dunn's method for pairwise multiple comparison. Median values and 75th and 25th percentiles are illustrated in figures. Results were considered significant at $P < 0.05$ value.

Results

Hemodynamic changes

Apart from an abrupt decrease during ischemia (because zero flow and pressure during occlusion in the affected intestinal area), no statistically significant differences between the groups or in comparison with baseline values could be detected in mesenteric vascular resistance (Fig. 2).

Changes in intramucosal pH

No difference in pHi values could be detected at baseline; neither 7-NI nor IPC influenced this parameter (Fig. 3). In response to 60 min of ischemia, however, a steep decrease in pHi could be detected ($P < 0.05$) in all of the groups, which was followed by a gradual recovery. Restoration in this parameter appeared to be more rapid in both IPC groups as pHi values were significantly higher than in the IR group during the 30th–90th min of reperfusion. At the end of reperfusion (reperfusion 120 min), pHi values were significantly higher only in the IPC + IR group than those in the test ischemia (IR) group.

Small bowel motility changes

Small bowel motility was not influenced by 7-NI alone or IPC at baseline, but a transiently reduced motility was observed in the 7-NI-treated group immediately after the IPC procedure (Fig. 4); this vanished in 30 min (just before the beginning of ischemia). Ischemia alone did not induce any significant changes in this parameter, but the early reperfusion phase (30 min of reperfusion) was associated with an increased intestinal motility. This intestinal hypermotility was prevented completely by IPC (to a similar extent in both the vehicle-treated and 7-NI-treated groups), and at the 60th min of reperfusion, even lower motility values could be detected than those seen at baseline. By the end of 2 h of reperfusion, the motility index was normalized in all the groups.

Changes in plasma NO_x levels

In the IR group, no significant changes in NO_x values could be detected at any phase of the experiments (Fig. 5). A temporary increase was observed in the vehicle-treated IPC group in

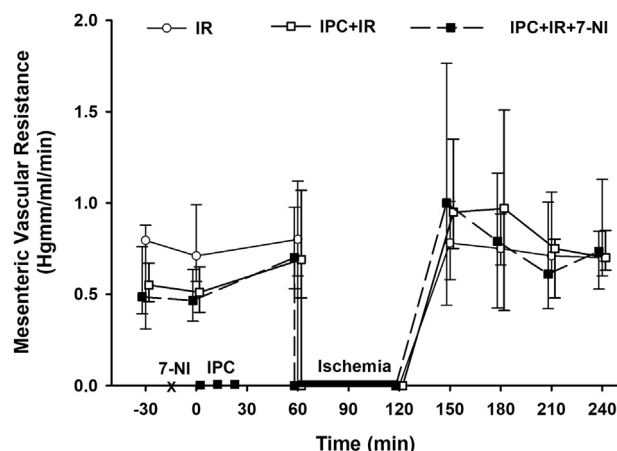


Fig. 2 – Time course of changes in mesenteric vascular resistance during baseline and during 60 min of SMA occlusion followed by 120 min of reperfusion (IR group). Further groups of animals were also subjected to IPC in the presence and absence of 7-NI treatment (IPC + IR + 7-NI and IPC + IR groups, respectively). Here, values are indicated as medians + 25th and 75th percentiles. Time-dependent differences from the baseline were assessed with Dunn's method. Differences between groups were analyzed with the Kruskal–Wallis (one-way analysis of variance on ranks) test, followed by Dunn's method for pairwise multiple comparison.

blood samples taken before the beginning of ischemia and in both IPC groups at the beginning (at 15 min) of reperfusion. This IPC-induced increase in NO_x levels, however, was missing in the 7-NI-treated IPC + IR group; furthermore, a marked increase in this parameter was seen at the last time point (120th min) of reperfusion.

Changes in ileal NOS activities

Ca²⁺-independent and Ca²⁺-dependent NOS activities were not significantly different between examined groups at baseline (Fig. 6A and B). As for Ca²⁺-independent NOS (iNOS) activity, no statistically significant changes could be detected throughout the entire experimental period in any of the groups (Fig. 6B). In the IR group, Ca²⁺-dependent NOS activity showed no changes during any phase of the entire examination period either. In the vehicle-treated IPC group, however, a marked increase in Ca²⁺-dependent NOS activity was found at the 15th min of reperfusion, but this increase was not present in the 7-NI-treated group.

Changes in ileal MPO activity

The baseline MPO activities were similar in the different groups (Fig. 7), but significant increases were seen at the 15th and 120th min of reperfusion in the IR group and at the 15th min of reperfusion in the 7-NI + IPC + IR groups. As compared with the IR groups, a significant reduction in MPO activity was evidenced in the IPC + IR group at both examined time points of reperfusion.

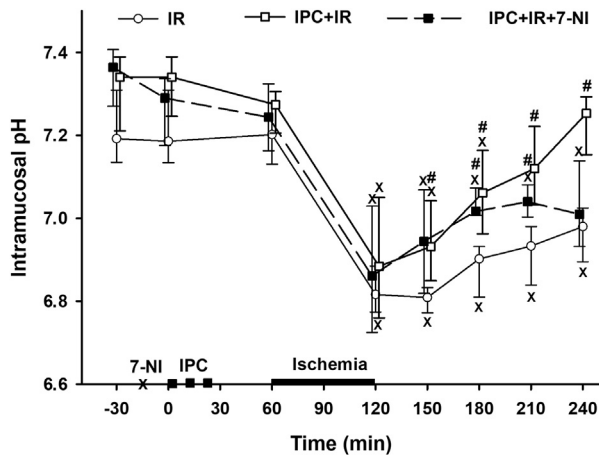


Fig. 3 – Time course of changes in intramucosal pH during baseline and 60 min of SMA occlusion followed by 120 min of reperfusion (IR group). Further groups of animals were also subjected to IPC in the presence and absence of 7-NI treatment (IPC + IR + 7-NI and IPC + IR groups, respectively). Here, values are indicated as medians + 25th and 75th percentiles. Time-dependent differences from the baseline were assessed with Dunn's method. Differences between groups were analyzed with the Kruskal–Wallis (one-way analysis of variance on ranks) test, followed by Dunn's method for pairwise multiple comparison. X represents $P < 0.05$ versus baseline, and # $P < 0.05$ versus IR.

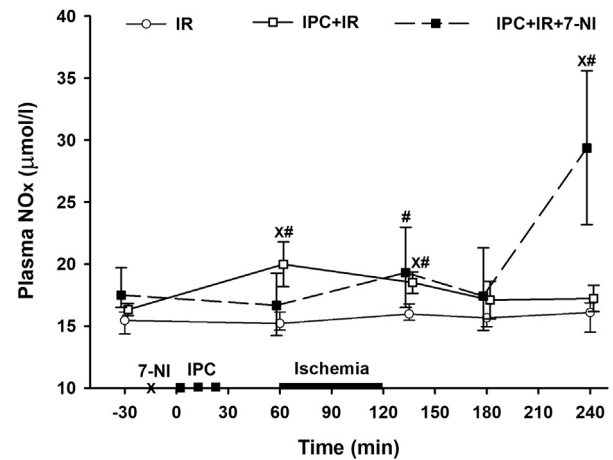


Fig. 5 – Time course of changes in plasma NO_x during baseline and during 120 min of reperfusion after a 60-min of SMA occlusion (IR group). Further groups of animals were also subjected to IPC in the presence and absence of 7-NI treatment (IPC + IR + 7-NI and IPC + IR groups, respectively). Here, values are indicated as medians + 25th and 75th percentiles. Time-dependent differences from the baseline were assessed with Dunn's method. Differences between groups were analyzed with the Kruskal–Wallis (one-way analysis of variance on ranks) test, followed by Dunn's method for pairwise multiple comparison. X represents $P < 0.05$ versus baseline, and # $P < 0.05$ versus IR.

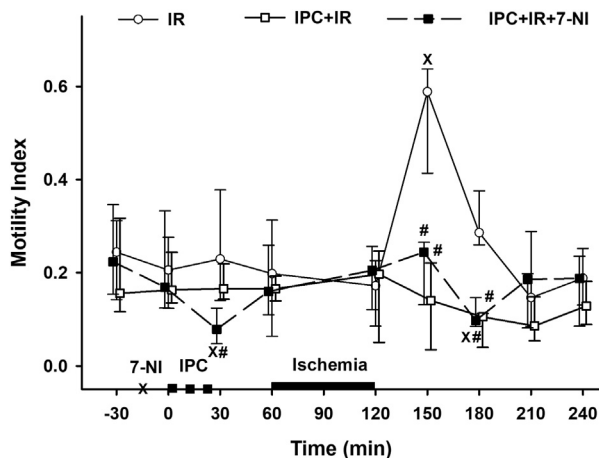


Fig. 4 – Time course of changes in motility index during baseline and during 60 min of SMA occlusion followed by 120 min of reperfusion (IR group). Further groups of animals were also subjected to IPC in the presence and absence of 7-NI treatment (IPC + IR + 7-NI and IPC + IR groups, respectively). Here, values are indicated as medians + 25th and 75th percentiles. Time-dependent differences from the baseline were assessed with Dunn's method. Differences between groups were analyzed with the Kruskal–Wallis (one-way analysis of variance on ranks) test, followed by Dunn's method for pairwise multiple comparison. X represents $P < 0.05$ versus baseline, and # $P < 0.05$ versus IR.

Mucosal injury and mast cell degranulation

Mast cell degranulation or mucosal injury was not present at baseline in any of the groups (Fig. 8A–D, Fig. 9A and B, Fig. 10A).

Fifteen minutes of reperfusion after a 60-min SMA occlusion caused a significant increase in mast cell degranulation (Fig. 8A, Fig. 9C and D) and mucosal injury (as evidenced by loss of enterocyte integrity and subepithelial changes; see details in legend for Figs. 9 and 10) and a reduction in mucosal thickness (Fig. 8C and D) in the IR and IPC + IR + 7-NI groups. These changes were exacerbated only moderately by the end of the observation period (120 min of reperfusion). In the IPC + IR group, however, a significantly lower extent of both mast cell degranulation and histologic injury were observed than in the other groups (Figs. 8 and 10C).

Discussion

These data confirm the results of previous studies^{2,5,6,25,26} on the protective effect of IPC in the intestine with the involvement of nNOS in the IPC-induced intestinal protection demonstrated here for the first time. The alleviating effects of IPC were marked by a more rapid recovery of intramucosal pH, restored IR-induced motility changes, and reduced local inflammatory reaction, including leukocyte accumulation and mast cell degranulation. As a result, IPC preserved mucosal integrity. Compared with rodents, pigs and dogs have similar innervation of the circular muscle layer to that of humans,¹⁹

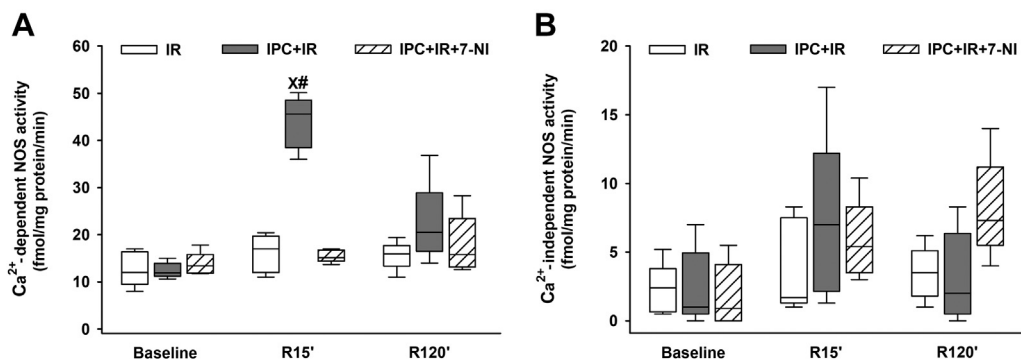


Fig. 6 – Changes in (A) constitutive NO synthase (Ca²⁺-dependent NOS) and (B) inducible NO synthase (Ca²⁺-independent NOS) activities in the affected ileal segment during baseline and at 15 and 120 min of reperfusion (IR). Further groups of animals were also subjected to IPC in the presence and absence of 7-NI treatment (IPC + IR + 7-NI and IPC + IR groups, respectively). Here, plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles. Time-dependent differences from the baseline were assessed with Dunn's method. Differences between groups were analyzed with the Kruskal–Wallis (one-way analysis of variance on ranks) test, followed by Dunn's method for a pairwise multiple comparison. X represents $P < 0.05$ versus baseline, and # $P < 0.05$ versus IR.

and it was possible to investigate IR- and IPC-induced changes in a complex and detailed fashion in this large animal model.

Reperfusion injury of the bowel affects different structural elements of the bowel in a particular sequence. The early reperfusion phase affects the integrity of the enterocyte lining and the rest of the mucosa relatively early, followed by injury of the muscle and neuronal structures.²⁷ In our study, the IPC-induced protection of the intestinal mucosal barrier (in

particular the villus tips) developed as early as the 15th min of reperfusion, which persisted over the examined 120-min reperfusion period. A similarly early (<120 min) protective effect was also demonstrated by others in rats as well.^{5,6,25} The mechanisms of protection provided by IPC in the intestine were strongly linked to oxidative stress^{4,5,28} but were also attributed to NO-dependent mechanisms.^{2,29} A transient peak in NO release was detected during the early postischemic phase in the rat intestine,² and this phenomenon was also observed if IR was preceded by IPC.³⁰ IR has been shown to trigger nNOS expression at the early stages of reperfusion³¹ even as early as 5 min of reperfusion *in vitro* (in Guinea pigs)³² or after 3 h of reperfusion *in vivo* in the rat jejunum.³¹ The effect of IPC on the expression and function of nNOS was examined here for the first time (at least to our knowledge). Specifically, an nNOS-dependent NO release can be presumed in response to IPC as levels of NO metabolites in the venous effluent of the intestine were effectively reduced when the specific NOS inhibitor 7-NI was administered before IPC. At the beginning (15th min) of reperfusion, the IPC-induced increase in Ca-dependent NOS activity was also prevented by 7-NI. We also saw a marked increase in NO_x metabolites occurring in the late phase of reperfusion (which is rather unexpected); this can probably be explained by nonenzymatic production of NO³³ resulting from hypoxia via impairment of the microcirculation in the postischemic bowel tissue.

Despite the previously observed debates,^{14–17} the contribution of nNOS to IPC-induced postischemic reactions is obviously positive in our study. Although the role of nNOS in the mechanisms of IPC was not examined elsewhere in the intestine, nNOS was also found to be protective in other organs, such as in heart IPC (both *in vitro* and *in vivo*).³⁴ This protection in the heart involves reduced oxidative/nitrosative stress, and the positive effects of IPC are lost after nonspecific NOS inhibition and in nNOS knockout animals.³⁵ Here, we could not examine the effect of nNOS inhibition on IR-induced changes (in adherence to the 3Rs approaches in animal research), but others also demonstrated a predominantly

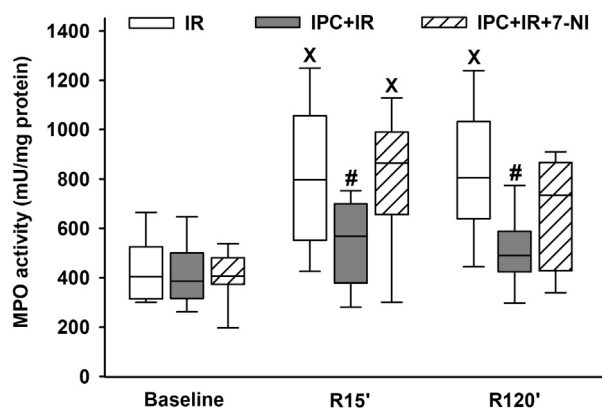


Fig. 7 – Changes in leukocyte accumulation (MPO activity) in the affected ileal segment during baseline and at 15 and 120 min of reperfusion (IR). Further groups of animals were also subjected to IPC in the presence and absence of 7-NI treatment (IPC + IR + 7-NI and IPC + IR groups, respectively). Here, plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles. Time-dependent differences from the baseline were assessed with Dunn's method. Differences between groups were analyzed with the Kruskal–Wallis (one-way analysis of variance on ranks) test, followed by Dunn's method for pairwise multiple comparison. X represents $P < 0.05$ versus baseline, and # $P < 0.05$ versus IR.

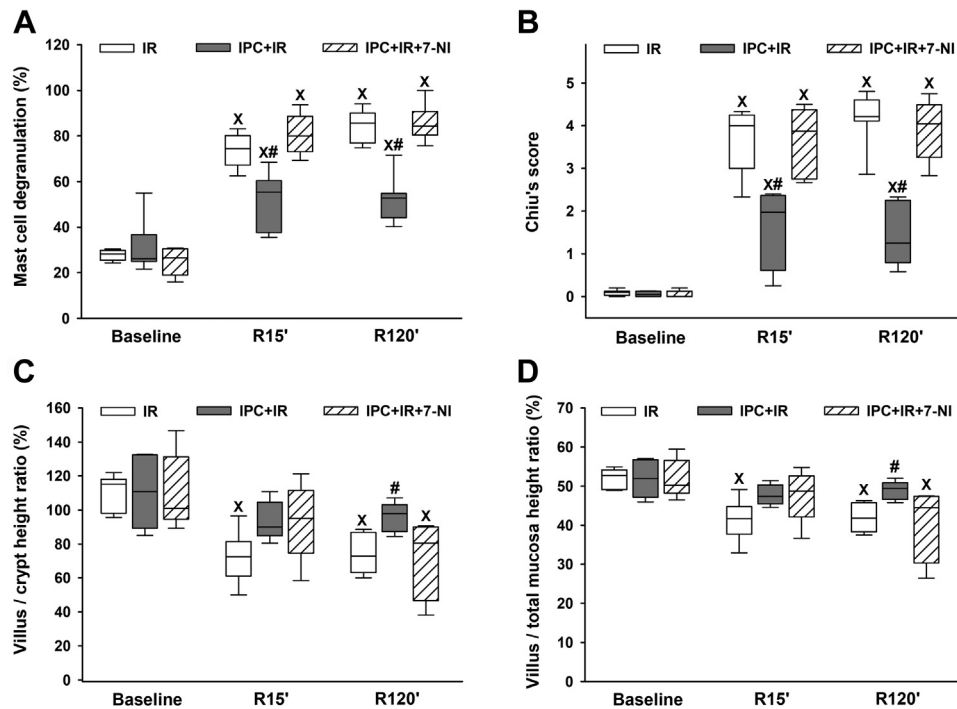


Fig. 8 – Changes in (A) MC degranulation, (B) mucosal histologic injury according to Chiu's grade, and mucosal height ([C] as expressed as villus/crypt height ratio and [D] villus/total mucosa height ratio) in samples taken from the affected ileal segment during baseline and at 15 and 120 min of reperfusion (IR). Further groups of animals were also subjected to IPC in the presence and absence of 7-NI treatment (IPC + IR + 7-NI and IPC + IR groups, respectively). Here, plots demonstrate the median (horizontal line in the box) and the 25th (lower whisker) and 75th (upper whisker) percentiles. Time-dependent differences from the baseline were assessed with Dunn's method. Differences between groups were analyzed with the Kruskal–Wallis (one-way analysis of variance on ranks) test, followed by Dunn's method for pairwise multiple comparison. X represents $P < 0.05$ versus baseline, and # $P < 0.05$ versus IR.

protective role of nNOS against intestinal reperfusion injury (with respect to bowel muscle contractility and polymorphonuclear leukocyte accumulation).³⁶

It appears that bowel IR may cause blood supply-dependent and nitrosative stress-mediated neuronal injury of the bowel, but this usually occurs after relatively long ischemic challenges (also depending on the species) and typically develops at later stages of reperfusion.^{7,8,37,38} Based on examinations of bowel motility in the present study, we found no major sign of neurogenic dysfunction of the bowel within the observed reperfusion period; furthermore, we noted a temporary postischemic enhancement in this regard at an early stage of reperfusion. On the other hand, because 7-NI was given before IPC, we had a chance to assess its direct effects (independently of IR). Interestingly, this manifested in an immediate, but temporary decrease in bowel motility. Because nNOS immunoreactive myenteric neurons are inhibitory motoneurons and descending interneurons, the above effect of 7-NI on bowel motility is surprising. It is known that NO, synthesized at a peripheral level by nNOS, reduces intestinal motility (as was demonstrated in the sheep, for instance)³⁹ via sustained smooth muscle hyperpolarization; this causes inhibition of spontaneous motility.⁴⁰ On the other hand, nonspecific inhibition of nNOS reduced gastrointestinal motility not only in our study but also elsewhere,^{8,41} and we

found a similar reducing effect of 7-NI on the colon motility in a subacute bowel obstruction model as well.¹³ In nNOS knockout animals, bowel motility (after 2 h of ischemia) did not decrease at 3 h but did so at 48 h of reperfusion,³⁶ whereas reduced intestinal transit time (i.e., increased motility) was observed under similar circumstances after nNOS inhibition.⁴¹ The effects of IPC- on IR-induced bowel motility changes were only examined in the long term, showing that IPC restores the reduced motility caused by 30 min of ischemia followed 6 h of reperfusion in rats.⁴² In our study, IPC prevented the IR-induced temporary increase in bowel motility at the early reperfusion phase, and 7-NI had no specific effect on this reaction within the examined time frame.

Although postischemic mucosa/enterocyte injury is believed to be linked mostly to intracellular free radical-mediated processes, bowel IR also induces marked inflammatory reactions (e.g., polymorphonuclear leukocyte accumulation and MC degranulation); these can also be inhibited by IPC.^{43,44} We saw simultaneous early increases in both tissue MPO and MC degranulation as early as 15 min after ischemia, which were both ameliorated by IPC. Similar, relatively early MPO changes were also demonstrated in rats (examined 30–60 min after ischemia).^{41,43} The nNOS dependency of the latter reaction was also proven as inhibition of nNOS (or KO)³⁶ reversed this protection. Interestingly, the

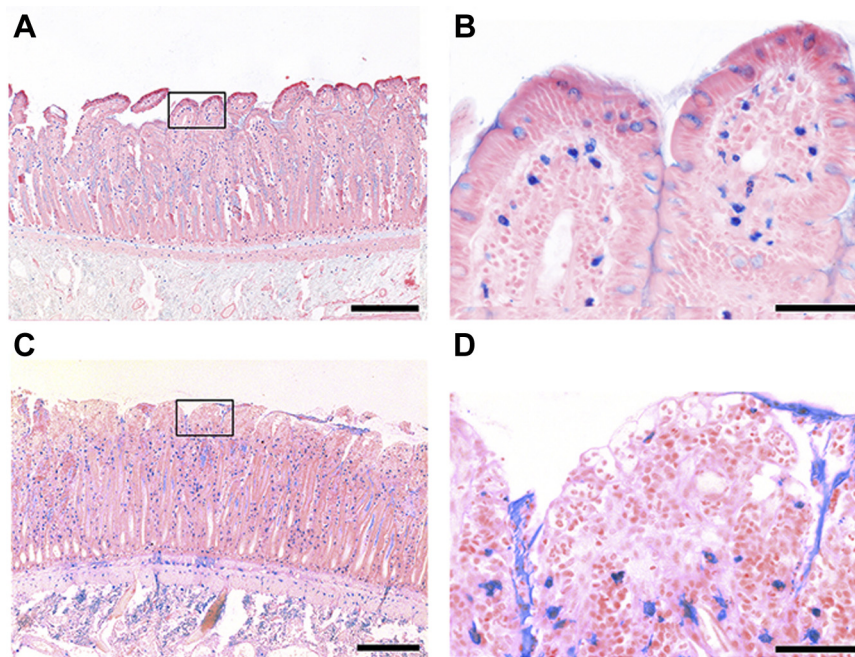


Fig. 9 – Representative micrographs showing mucosal MCs stained with Alcian blue and Safranin O at (A and C) lower (bar denotes 200 μ m) and (B and D) higher magnifications (bar denotes 50 μ m). In Panel B, please note intact intracellular granules, whereas Panel D shows a loss of intracellular granules and stained material dispersed diffusely and extracellularly as signs of MC degranulation at the 120th min of reperfusion in the IR group. (Color version of figure is available online.)

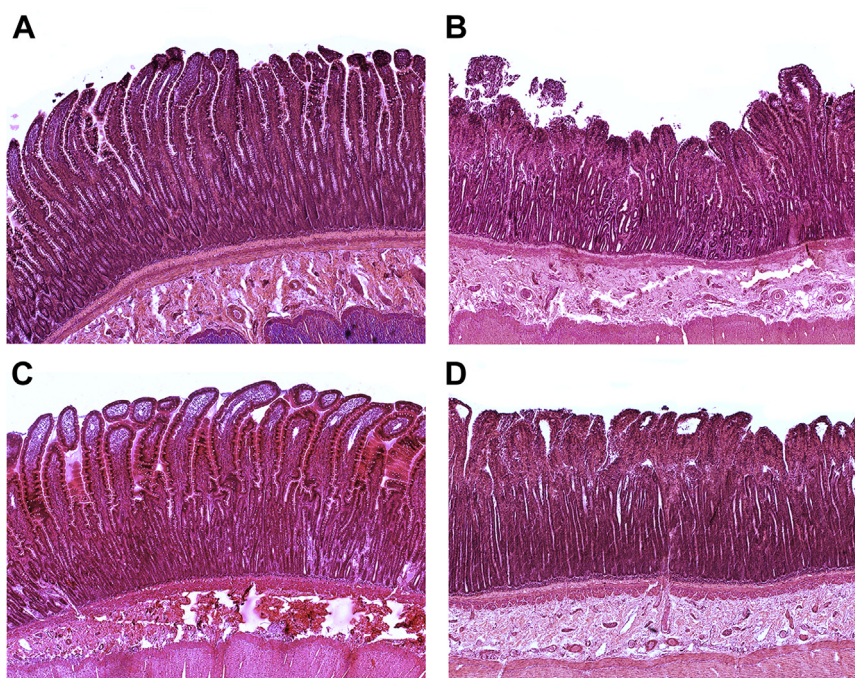


Fig. 10 – Representative micrographs of the ileal mucosa stained with hematoxylin and eosin staining at (A) baseline and (B) 120 min of reperfusion in the IR group, (C) the IPC + IR group, and (D) the IPC + IR + 7-NI group. Panels B and D demonstrate massive epithelial lifting down the side of villi, denuded villi, increased cellularity of the lamina propria, hemorrhage, and the end of the finger-like appearance of villous structures. Panel C displays the development of subepithelial Gruenhagen's space at the apex of the villus, capillary congestion, and extension of the subepithelial space with a lifting of the epithelial layer from the lamina propria. The bar denotes 200 μ m in all figures.

positive effect of IPC was also dependent on mast cell-dependent mediator release in the small bowel.⁴⁴ In our study, however, the IR-induced degranulation of MCs was not increased, but rather greatly prevented by IPC, which was reversed by 7-NI. It is therefore reasonable to assume that altered MC degranulation is a manifestation of reduced inflammatory reactions caused by nNOS in this IPC model, and this process may also be involved in mediating the structural injury of the intestinal mucosa.

Apart from oxidative injury, the integrity of the enterocyte lining is also highly dependent on adequate oxygen delivery. Similar to others (although working with rats),⁴⁵ our study found evidence of relatively minor IR-induced macrohemodynamic changes, but microvascular perfusion (as estimated indirectly by tonometry) underwent a marked and lasting deterioration. The positive effects of IPC on IR-induced deterioration of microvascular perfusion, tissue oxygenation, and leukocyte-endothelial interaction within the microvasculature of the small intestine are in evidence here and in a number of other studies.^{3,46,47} These reactions were also explained with an IPC-induced reduction of intestinal oxidative stress.²⁸ It appears that our study is the first to show that nNOS also plays a role in a more rapid microcirculatory recovery after bowel IPC. Because there was also remarkably early evidence of an increase in MPO and the appearance of morphologic injury in the reperfusion phase in our study, the importance of leukocyte-mediated reactions in preserving morphologic integrity (as another manifestation of nNOS-dependent IPC effects) cannot be ruled out either.

Conclusions

In the present study, the beneficial effects of intestinal IPC were examined in a relatively detailed fashion (with respect to pHi, motility dysfunction, leukocyte accumulation, mast cell degranulation, and mucosal injury). Changes in some of the examined parameters (in particular, in IPC-induced marked increase in NO availability: e.g., NO_x and constitutive NOS activity, pHi, inflammatory, and morphologic changes) could be influenced/reversed by pretreatment with the nNOS inhibitor 7-NI. Hence, our data strongly support the contribution of intestinal nNOS enzyme in the protective effect of IPC against the IR-related acute inflammatory response and morphologic injury in the small intestine.

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Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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