Studies on the mechanism of intestinal ischemic preconditioning – the role of neuronal nitric oxide synthase

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1. INTRODUCTION

Small bowel transplantation (SBTX) is a potentially life-saving solution for patients suffering from intestinal failure. Novel immunosuppressive strategies have greatly improved the clinical outcome of SBTX, but the survival of grafts is still poor due to non-immunological reactions caused by hypoxic damage or ischemia/reperfusion (IR) injury. Both warm ischemia and cold ischemic periods (i.e. SBTX) are characterized by severe biochemical and microcirculatory consequences which affect the integrity of the highly vulnerable mucosal barrier. These alterations may range from increased bowel permeability to translocation of gut-derived endotoxin and intraluminal bacteria, causing sepsis, multiple organ failure or death. The long-term consequences of SBTX also include intestinal motility changes caused by denervation during the surgical procedure. Some of the detrimental consequences of bowel IR injury could be greatly ameliorated by ischemic preconditioning (IPC), when transient, brief periods of ischemia followed by short intervals of reperfusion are applied prior to the final ischemic challange.

Since microcirculatory changes (e.g. patency of microvascular blood supply and inflammatory reactions) critically influence the integrity of the mucosal barrier during IR injury, our aim was to investigate the effect of IPC on these parameters in relation to SBTX. In the first studies, the consequences of local intestinal IPC were examined in a detailed fashion in a large animal model, where the bowel innervation is more similar to that in humans than that in smaller species. Nitric oxide (NO) has previously been implicated as a mediator in the mechanism of IPC, and neuronal NO synthase (nNOS) is a dominant NOS isoform in the gastrointestinal tract. Therefore, the aim of our further studies was to investigate the role of nNOS in IPC-induced protection after mesenteric IR using the selective enzyme inhibitor. Among the positive effects of IPC, NO availability, mucosal pH recovery, amelioration of polymorphonuclear leukocyte (PMN) accumulation and mast cell (MC) degranulation were detected and morphological injury was reduced by treatment.

2. MAIN GOALS OF THE STUDIES

- Our first objective was to compare the effects of IPC on the consequences of cold and warm IR during the early reperfusion phase in anesthetized dogs with respect to (1) local intestinal microcirculatory inflammatory and microvascular perfusion reactions, (2) potential denervation-related events, (3) involvement of MCs, and (4) morphological injury.
- Secondly, we set out to examine the possible involvement of nNOS in local IPC-induced protection in a surgically induced intestinal IR model in anesthetized dogs. To this end, 7-nitroindazole (7-NI), a specific inhibitor nNOS was administered prior to IPC. Changes in (1)

macro- and microhemodynamics, as well as in (2) PMN accumulation, (3) MC activation, (4) intestinal motility, (5) local NO production, and (6) structural injury were assessed.

3. MATERIALS AND METHODS

3.1. Experimental studies and protocols

The experiments were performed in 2 major studies on large animals (outbred dogs of either sex, body weight range: 12.2-17.3 kg). In both studies, the macro- and microcirculatory as well as biochemical effects of bowel IPC were examined. In Study 1, the above effects of IPC were examined in a small bowel autotransplantation model in dogs (Table 1). In the second study, the role of nNOS in the IPC-induced protection was assessed in a canine model of small bowel IR.

3.1.1. Experimental protocol of Study 1

Orthotopic SBTX was performed and the intestinal graft underwent 60 min cold ischemia, which was followed by 240 min of reperfusion (SBTX group, n=5). In another group, an identical experimental protocol was performed except that cold ischemia was preceeded by 3 cycles of 5-min ischemia/10-min reperfusion periods (IPC) 60 min prior to ischemia (IPC+SBTX group, n=5). The last groups served as sham-operated control (n=5). Leukocyte-endothelial cell interactions were monitored hourly by intravital videomicroscopy (IVM) during the reperfusion phase. An intravital orthogonal polarization spectral (OPS) imaging technique was used to monitor microvascular perfusion changes (functional capillary density (FCD), epithelial thickness and red blood cell velocity (RBCV)) and enterocyte injury in the intestinal villi before harvesting and hourly during the reperfusion phase after SBTX. Tissue biopsies from the affected bowel area were also taken for histological assessments of MC degranulation.

3.1.2. Experimental protocol of Study 2

In the first group, 60 min of ischemia was induced by occluding the superior mesenteric artery (SMA), which was followed by 120 min of reperfusion (IR). In the two other groups, IR was preceded by ischemic IPC, which was elicited by inducing 3 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 prior to IPC (IPC+IR+7NI 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 (NO_x) levels and NOS and myeloperoxidase (MPO) activities, as well as to assess MC degranulation and mucosal injury.

Table 1. A list of interventions and examined parameters in Studies 1 and 2

	Study 1	Study 2
Small bowel transplantation	+	
Duration of ischemia	60’	60’
Ischemic preconditioning	3x5’/10’	3x5’/5’
Reperfusion	4 hr	2 hr
Cardiac output measurements	+	
Superior mesenteric artery blood flow measurement	+	+
Assessment of PMN-endothelial interactions (IVM)	+	
Assessment of RBCV in capillaries of the villi (OPS)	+	
Assessment of intramucosal pH		+
Assessment of ileal motility		+
Plasma nitrate/nitrite measurement		+
Nitric oxide synthase activity measurements in the ileum		+
Assessment of morphological changes (villus tips; OPS)	+	
Assessment of morphological changes (histology: Chiu’s grade, villus height)		+
Assessment of mast cell degranulation (histology)	+	+

3.2. Hemodynamic and blood gas measurements; calculation of intramucosal pH (pHi) (Studies 1 and 2)

In both studies, the peripheral arterial and mesenteric venous pressure (via using Statham P23 Db transducers) as well as ultrasonic SMA flow (Transonic Systems Inc., Ithaca, NY, USA) measurements were performed. In Study 1, cardiac output was measured by thermodilution method. In Study 2, arterial blood gases and intramucosal pCO_2 (applying a silastic balloon catheter) were detemined with a blood gas analyzer (AVL, Graz, Austria) and pHi were calculated (using the modified Henderson–Hasselbach formula).

3.3. Intravital microscopic methods used to assess microcirculatory and morhologocal changes (Study 1)

Fluorescence intravital microscopy (IVM)

Microscope: Zeiss Axiotech Vario 100HD, 100W HBO mercury lamp, Acroplan 20× water immersion objective (Carl Zeiss GmbH, Jena, Germany), Camera: Teli CS8320Bi (Toshiba Teli Corporation, Osaka, Japan)

Labelling: rhodamine 6G-labelled PMNs (Sigma, St. Louis, MO, USA, 0.2%, 0.1 ml iv). Analysis: off line, IVM software package (Pictron Ltd., Budapest, Hungary)

Parameters: Adherent leukocytes (stickers) number of adherent cells per mm^2 of endothelial surface. Rolling leukocytes: the number of rolling cells/ number of the nonadherent leukocytes.

Orthogonal polarization spectral (OPS) imaging

Device: Cytoscan A/R, (Cytometrics, PA, USA)

Quantification: off line analysis, IVM software package (Pictron Ltd., Budapest, Hungary)

Parameters: FCD (relative length of perfused nutritive capillaries per observation area), RBCV, epithelial thickness

3.4. Intestinal motility measurements (Study 2)

Modified strain gauge transducer technique was applied, the transducers being sutured to the circular muscle layer of a terminal ileal segment. Motility index was determined by calculating the area under the motility curve as a function of time.

3.5. Plasma NOx level measurements (Study 2)

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

3.6. Measurement of NOS enzyme activity (Study 2)

NOS activity was determined in intestinal tissue biopsies based on the enzymatic conversion of ^{3}[H]-L-arginine to ^{3}[H]-L-citrulline. The Ca^{2+}-dependent and Ca^{2+}-independent NOS activities were assessed in the presence and absence of Ca-calmodulin, respectively.

3.7. Tissue MPO measurements (Study 2)

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

3.8. Determination of MC degranulation (Studies 1 and 2)

The percentage of degranulation (MC%) was calculated based on the number of intact (iMC) and degranulated (dMC) mast cells: MC% = dMC / (dMC+iMC) in 6-μm sections stained with Alcian blue and Safranin O.

3.9. Determination of morphological changes using histology (Study 2)

Method: Coded slides stained with hematoxylin-eosin in a blinded fashion

Parameters: mucosal damage: villus height – total mucosal height ratio (%), villus height – crypt height ratio (%), villus tip injury: using a modified version of Chiu's method.

3.10. Statistical analyses

In both studies, data analysis was performed using a statistical software package (SigmaStat 13.0 version for Windows, Jandel Scientific, Erkrath, Germany) with non-parametric 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.

4. RESULTS

Study 1

4.1. Macrohemodynamic effects of IPC during SBTX

Similarly, a significant cardiac index (CI) reduction was noted in the SBTX group during the reperfusion phase as compared to the baseline value or to the data of the sham-operated group. IPC before SBTX attenuated this change, and the improvement in CI was significant at 60 min and 240 min after reperfusion. In a comparison with the preharvesting level, a significant reduction in SMA blood flow was observed in the SBTX group throughout the entire reperfusion period, while IPC stabilized SMA blood flow at a significantly higher level, reaching similar values to those of the sham-operated group.

4.2. Effects of IPC on microcirculatory inflammatory reactions during SBTX

For technical reasons, IVM observations could not be performed before graft harvesting, and the baseline data for this parameter are therefore missing. The number of leukocyte-endothelial cell interactions was significantly higher after SBTX than in the sham-operated group. Reperfusion was followed by steady, approximately 70% primary (rolling) and an increasing number of secondary (sticking) leukocyte-endothelial cell interactions in the postcapillary venules of the mesentery. IPC significantly reduced the percentages of rolling and the number of firmly adhered white blood cells as well.

4.3. Microcirculatory effects of IPC during SBTX

SBTX and reperfusion induced a slight decrease in RBCV in the arterioles and venules of the villi. After IPC, there was no significant difference in these parameters as compared with the corresponding values in the SBTX group (data not shown). The villus capillary perfusion, quantified by FCD, was significantly decreased during reperfusion in the SBTX group, while this parameter stabilized at close to the control level in the IPC+SBTX group.

4.4. Effects of IPC on enterocyte injury during SBTX

The epithelial thickness, a marker of mucosal epithelial injury and epithelial integrity, decreased significantly and progressively during reperfusion after SBTX, compared to the baseline value

or to the data of the sham-operated group. The epithelial thickness in the IPC+SBTX group was reduced at 60 min relative to baseline, but thereafter it was unchanged, suggesting less severe epithelial structural damage.

4.5. Effects of IPC on MC degranulation during SBTX

No significant differences in mucosal MC degranulation were detected between the different experimental groups during baseline. In the SBTX group, cold ischemia alone caused significant increases in this parameter at the end of reperfusion. The cold ischemia-induced changes were significantly lower in the IPC+SBTX group at the end of reperfusion.

Study 2

4.6. Effects of 7-NI on IPC-induced macrohemodynamic changes during bowel IR

Neither 7-NI nor IPC caused any changes in the SMA flow during the preischemic period. Ischemia caused an abrupt decrease (due to zero flow during occlusion in the affected intestinal area) and early reperfusion (as measured at the 5^{th} min) a temporary increase in this parameter (hyperemia) in all of the groups under examination. During later stages of reperfusion, no differences in comparison with baseline values were detected. No significant differences were present in SMA flow between the groups at any stage of the experiment.

4.7. Effects of 7-NI on IPC-induced changes in pHi during bowel IR

No difference in pHi values was detected at baseline; neither 7-NI nor IPC influenced this parameter. In response to 60 min of ischemia, however, a steep decrease in pHi was detected ($P < 0.05$) in all of the groups, 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 30^{th}–90^{th} 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.

4.8. Effects of 7-NI on IPC-induced changes in small bowel motility during bowel IR

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; 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- and 7-NI-treated groups), and at the 60^{th} min of reperfusion, even lower motility values were detected than those seen at baseline. By the end of 2 hours of reperfusion, the motility index was normalized in all of the groups.

4.9. Effects of 7-NI on IPC-induced changes in plasma NO_x levels during bowel IR

In the IR group, no significant changes in NO_x values were detected at any phase of the experiments. A temporary increase was observed in the vehicle-treated IPC group in blood samples taken before the beginning of ischemia and in both IPC groups at the beginning of reperfusion (at 15 min). 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 end of reperfusion (at 120th min).

4.10. Effects of 7-NI on IPC-induced changes in ileal NOS activities during bowel IR

Ca^{2+}-independent and Ca^{2+}-dependent NOS activities were not significantly different between the groups under examination at baseline. As for Ca^{2+}-independent NOS (iNOS) activity, no statistically significant changes were detected throughout the entire experimental period in any of the groups. In the IR group, Ca^{2+}-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 Ca^{2+}-dependent NOS activity was found at the 15^{th} min of reperfusion, but this increase was not present in the 7-NI-treated group.

4.11. Effects of 7-NI on IPC-induced changes in ileal MPO activity during bowel IR

The baseline MPO activities were similar in the different groups, but significant increases were seen at the 15^{th} and 120^{th} min of reperfusion in the IR group and at the 15^{th} min of reperfusion in the IPC+IR+7-NI groups. As compared to the IR groups, a significant reduction in MPO activity was in evidence in the IPC+IR group at both time points of reperfusion under examination.

4.12. Effects of 7-NI on IPC-induced changes in mucosal injury and MC degranulation during IR

MC degranulation or mucosal injury was not present at baseline in any of the groups. Fifteen minutes of reperfusion after a 60-min SMA occlusion caused a significant increase in MC degranulation and in mucosal injury (as evidenced by loss of enterocyte integrity and subepithelial changes) and a reduction in mucosal thickness in the IR and IPC+IR+7NI 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 smaller extent of both MC degranulation and histological injury were observed than in the other groups.

5. DISCUSSION

5.1. Alleviating effects of IPC on cold and warm ischemia-induced circulatory and morphological injury of the small intestine in dogs

Issues of clinical relevance

The present studies were focused on an examination and modulation of the consequences of SBTX, which is a typical manifestation of surgically induced bowel ischemia. At the time of publication, our Study 1 was the first to provide a description of the microcirculatory consequences of IPC with SBTX. We observed the microcirculation directly with 2 different methods of intravital microscopy in a large animal (dog) model of SBTX. Cold graft perfusion, harvesting and vascular anastomoses were performed during autotransplantation to mimic clinical conditions but to exclude possible immunological complications (hence observing changes related to IR only). In this context, the therapeutic potential of IPC was tested and the role of nNOS examined in a surgically induced IR model (in Study 2).

Two manifestations of bowel IR examined in these studies

In Study 1, IPC was induced in an intestinal transplantation model, whereas in Study 2, the effects and mechanisms of IPC were examined in an bowel IR setting. There are certain differences between cold ischemia/SBTX and warm IR, which include (extrinsic) denervation of the bowel segment, transsection of the bowel walls and that of vessels and the lymphatic network, a somewhat longer duration of the operations and some protection which is derived from the cooling process in the case of SBTX. Although the examination period during reperfusion was somewhat different, we intended to compare findings for SBTX with those for IR only (as follows).

Macrocirculatory differences

One of the differences we detected between the SBTX and IR models is the different pattern of SMA blood flow changes. Firstly, a characteristic hyperemic flow response was observed in the IR groups at the onset of reperfusion, but it was not present in the SBTX groups (which underwent the same duration of ischemia). Secondly, IR was not accompanied by a considerable SMA flow reduction during reperfusion, whereas SBTX was accompanied by a severe postischemic deterioration. Both the absence of the hyperemic flow reaction and the reduced blood flow during reperfusion can be explained as a consequence of SBTX-induced denervation and its hemodynamic effect on local vascular regulation. Local (mesenteric) flow changes also showed parallel changes with those of CI (during the entire reperfusion period). Since there were no considerable changes in heart rate, cardiac preload (central venous pressure) or afterload (mean arterial pressure) observed (data not shown), probably a depressed heart contractility may mostly account for the reduced CI in the SBTX model. Mesenteric

ischemia affects a considerably large portion of the vascular bed; therefore, the remote effects (i.e. impaired cardiac function), which was previously interpreted as a "release of cardiodepressant substances" from the previously ischemic splanchnic vascular bed (or microvasculature), can be suspected. Another explanation behind these findings may originate in a vasoconstrictory reaction within the mesenteric microvasculature. Interestingly, most of these macrohemodynamic changes were beneficially influenced by IPC (restoring the SBTX/reperfusion-induced deterioration in both SMA flow and CI near the control values).

Microcirculatory differences

As shown by intravital microscopy, impairment in microcirculatory blood supply is a characteristic consequence of both IR and SBTX. Based on the present results, a long-term deterioration in microcirculatory blood flow is suspected in both models. In Study 1, there was a significant reduction in FCD, which was greatly ameliorated by IPC in the cold ischemia model of SBTX, whereas in Study 2, pHi, which provides an indirect measure of tissue oxygenation and therefore an estimation of the efficacy of microcirculatory blood flow, showed remarkably similar changes. It is important to note that IPC exerted similar protection (independently of the differences in cold/warm ischemia or denervation).

Both IR and SBTX induce marked enhancement of PMN-mediated reactions, and here IPC induced similar protection in both models (PMN-endothelial interaction in Study 1 and MPO activity in Study 2). As shown by Carmody et al., reduced PMN infiltration improved outcome of both warm IR and SBTX (with cold ischemia) in selectin knock out animals. In response to warm IR, increased MPO values were observed as early as the 15^{th} min of reperfusion in our model, but this phenomenon was observed similarly early by others after 6–24 hrs of cold ischemia in rats. Nonetheless, the duration of cold ischemia seems to influence the extent of adhesion molecule-dependent enhancement of PMN-endothelial interactions. With SBTX, IPC was shown to provide similar prevention of tissue infiltration of PMNs. It was suggested by de Groot H et al. that differences may occur in the inflammatory response following warm and cold ischemia: cold ischemia has been shown to affect endothelial cells to a greater extent than warm ischemia (via macrophages and neutrophils, and pro-inflammatory cytokine-dependent mechanism).

Potential denervation-related events

Denervation occurring during SBTX may also represent an important issue. Although the intrinsic nerve plexuses remain unaffected, external (e.g. sympathetic and parasympathetic) innervation of the bowel is lost, thus possibly also contributing to intestinal dysmotility. Interstitial Cajal cells were found to be temporarily impaired, and the myenteric and submucosal ganglia are also morphologically altered after SBTX. However, the spontaneous contractile

function recovers within approximately one month, while slow wave frequency recovers much later (within 18 months). It has been suggested that intestinal dysmotility leads to bacterial overgrowth due to the stasis of bowel contents in the long run. In other studies, the motility index decreased at the proximal colon after extrinsic denervation and exogenous NO caused a dose-dependent inhibition of spontaneous and basal contractile activity in intestinal muscle strips. In the present study, intestinal motility with SBTX was not examined, and apart from a transient postischemic increase in ileal motility, no further changes were in evidence in response to IR in the early reperfusion phase either.

Changes related to MC activation

In both of our studies (in the presence of both cold and warm IR), a similar extent of MC degranulation was in evidence. MC degranulation is known to be involved in IR injury in the rat small intestine, also modulating permeability changes both in dogs and rats. MCs facilitate mesenteric afferent signaling during acute intestinal ischemia as well. MC stabilizers (Na-cromoglycate and ketotifen) reduce bowel IR injury, e.g. Chiu's score, improve skin flap survival, also influencing MPO in rats, and ameliorate PMN-endothelial interactions in the feline and rat bowel. MC degranulation appears to be free radical-dependent in the rat intestine, since superoxide dismutase and catalase reduce this reaction. MC stabilization has been shown to increase survival in rats subjected to intestinal IR. Interestingly, the positive effect of IPC was dependent on MC-dependent mediator release in the small bowel and in the heart in rats.

The involvement of MCs in the cold IR-related processess has also been implicated elsewhere. Specifically, MCs may also be involved in acute rejection of renal allografts and at orthotopic liver transplantation. Through the secretion of both pro- and antiinflammatory mediators, MCs also influence the course and outcome in lung transplantation. Another aspect is the activatory effect of MCs on dentritic cells, which may have implications in transplantation biology. In our studies, a similar degree of MC degranulation was in evidence in response to IR and SBTX, and IPC alleviated these reactions to a similar extent. Based on the present findings, the role of MC-related processes in the mechanisms of IPC cannot be ruled out.

Morphological injury

In the present studies, mucosal injury showed a classical pattern and reached a similar extent in both studies and IPC again showed similar protection, but examination of the exact mechanism (e.g. whether it was caused by necrosis or apoptosis) was outside the scope of the studies. It has been shown that both pHi changes, and the extent of PMN transmigration show a good correlation with the overall severity of intestinal histological injury. It was suggested by Rauen U et al., however, that cold storage may induce apoptosis *per se* in several cell types (e.g. endothelial cells, hepatocytes and renal tubular cells) via cold-induced accumulation of iron

ions, which triggers an activation of mitochondrial apoptotic reactions (via their involvement in catalizing ROS formation).

Conclusions of Study 1

Using intravital microscopic and conventional histological methods, our present studies demonstrated that the reperfusion phase after warm IR and SBTX is associated with a similar extent of microcirculatory deterioration, a remarkably early and similar degree of PMN accumulation and MC degranulation as well as mucosal injury. The data also showed that these changes could effectively be ameliorated by IPC in both models. Based on the results of hemodynamic assessment, however, differences between warm and cold IR was also suspected (i.e. differeces in postischemic flow changes), but the effect of IPC appeared to be similar.

5.2. The role of nNOS in intestinal IPC-induced protection against warm bowel ischemia in dogs

Our second study confirms the results of previous studies on the protective effect of IPC in the intestine, but the involvement of nNOS in IPC-induced intestinal protection was first demonstrated here. This conclusion was derived from findings where inhibition of nNOS with 7-NI antagonized the protection provided by IPC in numerous respects (pHi, intestinal motility, PMN accumulation, MC degranulation and morphological injury).

Time course of mucosal injury during reperfusion

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 to the muscle and neuronal structures. In our study, the IPC-induced protection of the intestinal mucosal barrier (in particular the villus tips) developed as early as the 15^{th} min of reperfusion, which persisted over the 120-min reperfusion period under examination. In other studies (performed on rats), IPC exerted marked protection of intestinal mucosa (in particular villus tips), which manifests in reduced mucosal permeability and apoptosis in the early reperfusion phase (<120 min) and in enhanced IPC-induced mucosal regeneration in the later phase. The mechanisms of protection provided by IPC in the intestine were strongly linked to oxidative stress (MDA and TNF-α release; endothelial activation and adhesion molecule release) but were also attributed to NO-dependent mechanisms. A transient peak in NO release was detected during the early postischemic phase in the rat intestine, and this phenomenon was also observed if IR was preceded by IPC.

NOS activation during reperfusion

IR, however, has also been shown not only to reduce, but also to trigger nNOS expression at early stages of reperfusion even as early as 5 min of reperfusion *in vitro* (in Guinea pigs) or after 3 h of reperfusion *in vivo* in the rat jejunum. The effect of IPC on the expression and

function of nNOS was first examined here (at least to our knowledge). Specifically, a 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 (15^{th} min) of reperfusion, the IPC-induced increase in Ca^{2+}-dependent NOS activity was also prevented by 7-NI. Further, we 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 non-enzymatic production of NO resulting from hypoxia via impairment of the microcirculation in the postischemic bowel tissue. It remains unclear, however, why these changes did not manifest in alterations in intestinal motility or microcirculation (see later).

The role of nNOS (similarly to that of NO in general) is usually found, however, to be rather controversial (in relation to both reperfusion and IPC) by others. Rivera LR et al. have also discussed this issue and suspected that, despite some extent of free radical production derived from nNOS, this NOS isoform has a predominantly protective role of nNOS against intestinal reperfusion injury (with respect to bowel muscle contractility and PMN accumulation). The same group found that nitrosative stress takes place mostly in a later phase of reperfusion in the enteric nerve system and in the muscle layer, which is obviously preceded by damage to the mucosa. Furthermore, genetic ablation of the nNOS gene worsens the IR-induced bowel injury (inflammatory infiltration and motility). So far, the role of nNOS in IPC has been examined to a lesser extent. Despite the previously observed debates, 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*). 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.

Bowel motility changes during reperfusion

It appears that bowel IR may cause blood supply-dependent and/or 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. 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, since 7-NI was administered 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. Since 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 sheep, for instance) via sustained smooth muscle hyperpolarization; this causes inhibition of spontaneous motility. On the other hand, nonspecific inhibition of nNOS reduced gastrointestinal motility not only in our study, but also elsewhere, and we found a similar reducing effect of 7-NI on the colon motility in a subacute bowel obstruction model as well. We also observed a temporary increase in bowel motility at the 30th min of reperfusion which was not seen in either of the IPC groups. Early postischemic changes in bowel motility was not examined by others with similar methods, but reperfusion after a 1 hr segmental ileal ischemia followed by 2 hrs of reperfusion was similarly associated with reduction in motility (as measured by intestinal transit time).

The effects of IR on motility are usually examined in a longer run, and the results are somewhat controversial. 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, while reduced intestinal transit time (i.e. increased motility) was observed under similar circumstances after nNOS inhibition. 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. 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.

PMN- and MC-related changes

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. PMN accumulation and MC degranulation); these can also be inhibited by IPC. Increased PMN accumulation (i.e. increased tissue MPO) in the affected intestine after IR was also demonstrated elsewhere as early as 30–60 min after ischemia in rats. 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), and this effect was also present at 6 hrs of reperfusion. The nNOS dependency of the latter reaction was also proven as inhibition of nNOS (or KO) reversed this protection. In a cremaster flap model, 7-NI did not influence the effect of IPC on PMN-endothelial interactions (4h/2h IR). Our study is the first to show that nNOS also plays a role in the mechanism of protection provided by IPC in reducing MPO activity in the intestine.

Interestingly, the positive effect of IPC was also dependent on MC-dependent mediator release in the small bowel. 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. Similarly to others (though working with rats), 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. These reactions were also explained with an IPC-induced reduction of intestinal oxidative stress. 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. Since there was also remarkably early evidence of an increase in MPO and the appearance of morphological injury in the reperfusion phase in our study, the importance of leukocyte-mediated reactions in preserving morphological integrity (as another manifestation of nNOS-dependent IPC effects) cannot be ruled out either.

Conclusions of Study 2

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, MC degranulation and mucosal injury). Changes in some of the parameters under examination (in particular in IPC-induced marked increase in NO availability: e.g. NO_x and constitutive NOS activity, pHi, and inflammatory and morphological changes) could be influenced/reversed by pre-treatment with the nNOS inhibitor 7-NI. Hence, our data strongly supports the contribution of intestinal nNOS enzyme in the protective effect of IPC against the IR-related acute inflammatory response and morphological injury in the small intestine.

6. SUMMARY OF NEW FINDINGS

1. Using IVM, our experimental study was the first to demonstrate the beneficial postischemic microcirculatory effects of IPC after cold bowel ischemia in a large animal model of SBTX.
2. We have shown similar microcirculatory deterioration, early PMN accumulation, MC degranulation and mucosal injury in both IR and SBTX models and have demonstrated that these changes can be ameliorated by IPC. The early postischemic intestinal flow changes after SBTX are due to cold ischemia and denervation.
3. IPC before IR induces an acute release of NO and an increase in the Ca^{2+}-dependent intestinal NOS activity during the early reperfusion phase.
4. IPC reduces the IR-induced temporary increase in intestinal motility in the early reperfusion phase and protects against IR-related acute inflammatory response and intestinal damage characterized by amelioration of microcirculation/tissue oxygenation, PMN accumulation, MC degranulation and structural injury.
5. The mechanism of intestinal IPC-induced organ protection is mediated through an effect on nNOS-linked NO generation.

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