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

Sándor Varga M.D.

Ph.D. Thesis

Doctoral School of Multidisciplinary Medical Science

Institute of Surgical Research
Faculty of Medicine, University of Szeged

Supervisors:
Andrea Szabó M.D., Ph.D.
József Kaszaki Ph.D.

Szeged
2019

LIST OF FULL PAPERS RELATED TO THE SUBJECT OF THE THESIS

I. Wolfárd A, Kaszaki J, **Varga S**, Lázár G, Boros M. Early microcirculatory changes after ischemic preconditioning and small bowel autotransplantation. Eur Surg Res. 2007;39:284-290. **IF: 0.92**

II. **Varga S**, Juhász L, Gál P, Bogáts G, Boros M, Palásthy Z, Szabó A, Kaszaki J. Neuronal nitric oxide mediates the anti-inflammatory effects of intestinal ischemic preconditioning. J Surg Res. 2019;244:241-250. **IF: 1.872**

LIST OF ABSTRACTS RELATED TO THE SUBJECT OF THE THESIS

I. **Varga S**, Krishnan V, Balázs L, Gál P, Kaszaki J, Boros M. The role of neuronal nitric oxide in the mechanism of intestinal preconditioning. Eur Surg Res. 2002;34(S1): 2-3.

II. Wolfárd A, Kaszaki J, Szalay L, **Varga S**, Balogh Á, Boros M. Effects of ischemic preconditioning on the graft microcirculation during small bowel transplantation. Eur Surg Res. 2002;34(S1):17.

III. Wolfárd A, Kaszaki J, Szalay L, **Varga S**, Balogh Á, Boros M. Ischemic preconditioning improves graft microcirculation during small bowel transplantation. Acta Physiol Hung. 2002;89:65.

IV. Kaszaki J, **Varga S**, Gál P, Nagy S, Boros M. Neuronal nitric oxide protects the mucosa following intestinal preconditioning. Acta Physiol Hung. 2002;89:119.

V. Wolfárd A, Kaszaki J, Szalay L, **Varga S**, Balogh Á, Boros M. Ischemic preconditioning improves macrohemodynamics and graft microcirculation after small bowel transplantation. Shock. 2002;18S:74.

VI. **Varga S**, Kaszaki J, Balázs L, Gál P, Lőrincz A, Nagy S, Boros M. Protective role of neuronal nitric oxide following intestinal preconditioning. Shock. 2002;18S:27.

VII. **Varga S**, Kaszaki J, Balázs L, Gál P, Bogáts G, Boros M. A neuronális eredetű nitrogénmonoxid szerepe az ischaemiás prekondicionálásban. Magy Seb. 2003;56:158.

VIII. Wolfárd A, Kaszaki J, **Varga S**, Balogh Á, Boros M. A vékonybél mikrokeringésének változásai ischaemiás prekondicionálás hatására vékonybél autotranszplantációt követően – a hízósejtek szerepe. Magy Seb. 2003;56:159.

LIST OF ABBREVIATIONS

7-NI	7-nitroindazole
CI	cardiac index
cNOS	Ca^{2+}-dependent NOS
eNOS	endothelial NOS
FCD	functional capillary density
GSH	glutathione
ICAM-1	intercellular adhesion molecule-1
iNOS	inducible NOS
IPC	ischemic preconditioning
IR	ischemia/reperfusion
IVM	intravital videomicroscopy
LDH	lactate-dehydrogenase
L-NAME	N(ω)-nitro-L-arginine methyl ester
MC	mast cell
MDA	malondialdehyde
MPO	myeloperoxidase enzyme activity
NF-κB	nuclear factor-kappa B
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NO_x	nitrate/nitrite
OPS	orthogonal polarization spectral
pHi	intramucosal pH
PMN	polymorphonuclear leukocyte
RBCV	red blood cell velocity
ROS	reactive oxygen species
SBTX	small bowel transplantation
SMA	superior mesenteric artery
SOD	superoxide dismutase
TNF-α	tumor necrosis factor alpha
VCAM-1	vascular adhesion molecule-1
XOR	xanthine oxidoreductase

CONTENTS

SUMMARY

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.

Ischemic preconditioning (IPC) involves transient, brief periods of ischemia, followed by short intervals of reperfusion prior to the final ischemic challenge. Some of the detrimental consequences of non-immune-mediated bowel injury can be ameliorated by IPC, but the details of the underlying, protective mechanisms are still largely unknown.

Since microcirculatory reactions can affect the blood supply as well as microvascular inflammatory reactions and can critically influence the integrity of the mucosal barrier during IR injury, major emphasis was put on the effect of IPC on these changes in relation to SBTX in the present studies. Firstly, the consequences of local intestinal IPC were examined in a detailed fashion in a large animal model, where bowel innervation is more similar to that in humans than in smaller species. In this system, we demonstrated that IPC improves macrohemodynamics and intestinal microcirculation and reduces leukocyte-mediated tissue injury during reperfusion. 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 7-nitroindazole. Among the positive effects of IPC, NO availability, mucosal pH recovery, amelioration of leukocyte accumulation and mast cell degranulation were detected, and morphological injury was reduced by the treatment. Therefore, it was concluded that IPC represents an effective tool to ameliorate reperfusion injury during SBTX, and the data indirectly proved that NO generated by intestinal nNOS plays a pivotal role in IPC-linked tissue protection during IR injury.

1. INTRODUCTION

1.1. Clinical indications and complications of small bowel transplantation (SBTX)

One of the main indications of small bowel transplantation (SBTX) is to overcome the negative consequences of short bowel syndrome. Short bowel syndrome is a manifestation of "intestinal failure", which is defined as an inability of the gastrointestinal tract to meet the nutritional demands of the body (Kappus M 2016). This often life-threatening condition (O'Keefe SJ 2006) is caused by numerous intestinal diseases, including Crohn's disease, mucosal defect, fistulae, vascular complications, radiation enteritis, intestinal obstruction, dysmotility, and congenital defect or may evolve secondarily to surgical resection. The resultant malnutrition typically necessitates temporary or permanent dependence on parenteral nutrition in these patients. Apart from bowel lengthening procedures, improved enteric absorptive surface area can be provided via SBTX for patients with severe complications of the underlaying intestinal disease or of long-term parenteral nutrition therapy (Loo L 2017).

The first human SBTX was conducted in 1967 (Lillehei RC 1967) several years after preclinical and animal studies (Lillehei RC 1959; Starzl TE 1960). Today it is recognized that appropriate patient selection should be ensured because of complications related to surgery and the necessity of long-term immunosuppressive therapy. Although novel immunosuppressive strategies have greatly improved the clinical outcome of SBTX, survival of grafts (and thus patients) is still poor and the results markedly worse than the success rates for the transplantation of other organs (e.g. kidney, liver or heart) (Legendre C 2014; Neuberger J 2016; McCartney SL 2017). This is mainly due to non-immunological reactions, such as hypoxic damage or ischemia/reperfusion (IR) injury, which play pivotal roles in postoperative graft dysfunction and damage (Mallick IH 2004).

1.2. Manifestations and mechanisms of ischemia/reperfusion (IR) injury of the bowel

Within the intestinal mucosal villi, a single-cell epithelial layer of enterocytes forms a barrier between the internal milieu and the external environment; therefore, injury to this rather vulnerable compartment manifests in changes in bowel permeability, leading to detrimental consequences, such as translocation of gut-derived endotoxin and intraluminal bacteria (Gayle J 2002; Szabó A 2006), sepsis and eventually multiple organ failure. Mucosal barrier dysfunction often occurs due to the consequence of hypoxia caused by vascular occlusion (arterial or venous thrombosis and embolism) or hypoxia/reoxygenation injury, which also evolves during bowel surgery. Temporary ischemia *per se* induces the

oxygen and energy deficit in the affected tissues (particularly in the villus typs), which is followed by reperfusion-induced tissue damage, frequently exceeding the conseqences of the ischemic insult itself (Stallion A 2002; Mallick IH 2004).

Although restoration of the blood supply is essential for tissue survival, the reperfusion phase is characterized by an immediate and harmful production of reactive oxygen species (ROS). This originates from sequential, univalent reductions of molecular oxygen, leading to the formation of superoxide radicals which are subsequently converted to hydrogen peroxide and hydroxyl radicals (Parks DA 1982; Parks DA 1983; Granger DN 1986). Hydroxyl radicals then cause lipid peroxidation of cell membranes, which brings about increased production and systemic release of proinflammatory eicosanoids. The enzyme initiating this reaction is xanthine oxidoreductase (XOR), particularly abundant in the gut (Parks DA 1988). The XOR-derived superoxide anion can also react with nitric oxide (NO) to form peroxynitrite, which is also a reactive promoter of lipid peroxidation and cellular membrane damage (Beckman JS 1996). Since ROS also cause endothelial activation, some of the subcellular biochemical changes are closely related to deterioration in microcirculation in different organs, including the bowel (McCord JM 1985; Schoenberg MH 1993; Menger MD 2000; Dejana E 2009; Vollmar B 2011; Park-Windhol C 2016). Consequently, impaired endothelium-dependent vasodilation and increased microvascular permeability are among the most important consequences of ROS and peroxynitrite formation in the gut (Björk J 1982; Parks DA 1983; Beckman JS 1996). As shown by intravital videomicroscopy (IVM), incomplete restoration of local microcirculatory blood flow (partially due to endothelial injury in the intestinal villi) can also be observed as a consequence of IR injury (Guan Y 2009). Furthermore, tissue damage leads to an enhanced release of proinflammatory cytokines (e.g. interleukins, platelet activating factor and tumor necrosis factor alpha (TNF-α)) (Granger DN 1988; Khan BV 1996; Pober JS 2007). These cytokines cause an increased expression of adhesion molecules responsible for the interactions between endothelial cells and polymorphonuclear leukocytes (PMNs) (Pohlman TH 1986; Sugama Y 1992; Panés J 1999; Pober JS 2007). Nicotinamide adenine dinucleotide phosphate-oxidase located in the plasma and phagosomal membranes of PMNs, is also an important source of ROS formation, leading to further propagation of tissue and microvascular injury (Rybicka JM 2010).

Importantly, a correlation between the number of recruited PMNs and a high extent of tissue damage was also demonstrated (Harris AG 1996). The adhesion of PMNs directly induces ROS formation in the postcapillary venules (Granger DN 1986; Suzuki M 1989,

1991; Marui N 1993; Khan BV 1996; Lee S 2007), together with platelet−leukocyte adhesion (Cooper D 2004; Beuk RJ 2008) and platelet aggregation (Iuliano L 1994; Leo R 1997; Iba T 2006). These changes further lead to the transmigration of recruited PMNs from the endothelial surface of the lumen to the surrounding tissues across the postcapillary venules (Grisham MB 1998; Ley K 2007; Woodfin A 2011; Muller WA 2011; Cerutti C 2017; Schimmel L 2017). ROS promote further activation of PMNs, which enhances inflammatory reactions in the microcirculation (Grisham MB 1986; Wankowicz Z 1988; Cook-Mills JM 2011).

1.3. Characteristics of reperfusion injury during SBTX

Among the biochemical consequences of SBTX, oxidative reactions were demonstrated after cold IR, similar to those seen with warm IR in a canine model, and these were reduced by conventional preservation solutions (Ferencz A 2002). Reperfusion after SBTX was also associated with a progressive decrease in mesenteric blood flow, an increase in mesenteric vascular resistance as well as enhanced accumulation and ROS production of PMNs (Wolfárd A 1999). It has also been proposed that these leukocyte-mediated postischemic microvascular reactions also play an important role in reperfusion injury after cold ischemia in small bowel grafts (Gonzalez AP 1994). To study these *in vivo* reactions, intravital fluorescence microscopy can be applied to assess this leukocyte–endothelial cell interaction that causes microvascular reperfusion injury in SBTX in rats (Gonzalez AP 1994) and in dogs (Wolfárd A 2002), whereas orthogonal polarization spectral (OPS) imaging technique can also be used to visualize changes in mucosal morphology and epithelial thickness (Wolfárd A 2002). Furthermore, a motoric dysfunction in the graft was observed in rats with the involvement of muscularis macrophages during the course of the inflammatory processes (Schaefer N 2007).

Although rodents are easily accessible and frequently used animals in such studies (because of a relatively wider range of tests available to detect various inflammatory markers), these models also have many obstacles. The gastrointestinal tract of larger animals (e.g. pigs and dogs) and especially the innervation of the circular muscle layer of these species have more similarities to those of humans than of smaller species (Furness JB 2014). For this reason, many earlier studies involved the use of dogs, particularly if examination of the long-term consequences of SBTX were concerned. As such, functional intestinal changes (e.g. dysmotility and differently affecting distinct small intestinal regions) were demonstrated in dog models 2 and 8 weeks after denervation (Zyromski NJ 2002), which was followed by a jejunal cholinergic and adrenergic smooth muscle hypersensitivity phase 2 weeks after

surgery (Balsiger BM 2003). Furthermore, altered intestinal transit time (delayed gastroduodenal emptying), reduced intestinal flow rates (Johnson CP 2001) and impaired absorption were attributed to the presence of intestinal denervation in dogs after 4–12 weeks (Ishii H 1993; Tsiotos GG 2001). Diarrhea caused by malabsorption of water and electrolytes as well of the effect of altered bile salt secretion were also observed as a result of denervation 8 weeks after SBTX in dogs (Oishi AJ 1995). Plasma levels and composition of gastrointestinal hormones were similarly shown to be moderately altered after SBTX in similar canine models after 2–12 weeks (Nelson DK 1993; Adrian TE 1994).

1.4. Nitric oxide (NO) production in the gut

Being a highly effective vasodilator molecule, NO is an important intrinsic regulator of vascular tone under both healthy and diseased conditions. As for the gut, the mucosal blood flow, oxygen uptake and intestinal motility (Brookes SJ 1993) are simultaneously determined by NO-dependent mechansims (Alemayehu A 1994; Salzman AL 1995; Qu XW 2005; Nair J 2019). At a microcirculatory level, NO has important anti-adhesive characteristics (Gaboury J 1993). The inhibition of constitutive forms of NO synthesis evokes the recruitment of adherent PMNs and causes intestinal mucosal damage (Kubes P 1991, 1993b, 2000). During IR challenge, NO donors and NO-releasing compounds (e.g. SIN-1, sodium nitroprusside, CAS 754 and FK409) markedly attenuate IR-induced tissue injury and preserve mucosal barrier function in many species (Qu XW 1999a, 1999b; Emre A 2008), including cats (Kanwar S 1994b) and dogs (Kawata K 2001; Payne D 1993). Similarly, blockade NO synthesis exacerbates the consequences of small bowel IR (e.g. mucosal barrier dysfunction), and this effect was reversed by L-arginine administration (Kubes P 1993a). On the other hand, the contribution of NO-derived peroxynitrite to tissue injury in the postischemic intestine was also demonstrated (Cuzzocrea S 1998).

In the gastrointestinal tract, three endogenous isoforms of nitric oxide (NO) synthase (NOS), neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS) are responsible for NO production (Qu XW 1999b). In all three cases, the biosynthesis pathways use L-arginine, molecular oxygen and, additionally, nicotinamide adenine dinucleotide phosphate (NADPH) and tetrahydrobiopterin (BH4), leading to the production of NO and L-citrulline (Knowles RG 1994). These isoforms have different calcium dependence: eNOS and nNOS are Ca^{2+}-dependent, while iNOS is Ca^{2+}-independent (Alderton WK 2001). Among the two Ca^{2+}-dependent constitutive isoforms, neuronal NOS (nNOS or type I NOS) is a predominant isoenzyme in the rat small intestine (Qu XW 1999a, 1999b). Similar isoenzyme distribution was demonstrated in later experimental studies in

the small intestine in large animals as well. As such, a marked pharmacological inhibition of the constitutive enzyme activity with 7-nitroindazole (7-NI) (Moore PK 1996) (by 40–70% from the oral to the aboral direction, respectively) was achieved in the colon in a canine model of bowel obstruction, suggesting a relatively high activity of nNOS in the gastrointestinal tract (Palásthy Z 2006).

Interestingly, stable end-products of NO (nitrite and nitrate) were primarily found on the apical side of intestinal epithelial cells (Witthöft T 1998). NO has also been shown to influence tight junction protein expression of intestinal epithelial cells and to regulate their function via free radical-dependent mechanisms (Mu K 2019). This latter effect may be based on numerous mechanisms, including the scavenging effect of lipid or protein radicals, and on hydrogen peroxide-induced changes in the protein tyrosine phosphorylation of enterocytes (Katsube T 2007), by altering the glutathione (GSH/GSSG)-related effects on phosphatases and protein tyrosine phosphorylation, and finally via redox change-induced effects on enzymes (Schafer FQ 2001; Gow AJ 2004; Tegeder I 2019).

It appears that bowel IR is associated with damage to NOS-positive neurons (swelling and distortion) (Rivera LR 2009, 2011b) in small animal models and, in parallel, with changes in gastrointestinal motility (notably a slowing of transit) (Udassin R 1995; Grossie VB Jr 2001; Türler A 2002; Hakgüder G 2002). These changes (complete damage to these neurons) can be detected after 2 days. Apart from structural injury, nitrosylation of proteins via a peroxynitrite-dependent mechanism may also represent a manifestation of IR injury (Ferrer-Sueta G 2009). These peroxynitrite-related events via nitrosylate tyrosine of proteins may also contribute to the function of proteins (including NOS). Further, NO may play a role in cytotoxic damage to neurons as a result of increased cytoplasmic Ca^{2+}, which triggers excess NO production by the Ca^{2+}-dependent NOS isoforms (Dong Z 2006). Although S-nitrosylation is a physiological mechanism of the signal transduction of NO, excess production of nitrosylate tyrosine residues or cysteine nitrosothiols can contribute to protein misfolding and eventually to neuronal damage (Stamler JS 1992; Nakamura T 2007; Foster MW 2009; Savidge TC 2011; Rivera LR 2011a, 2011b).

It appears that intestinal IR injury evokes an acute inflammatory response, and the role of nNOS in this scenario remains controversial. Several groups have attributed a pro-inflammatory role to nNOS (Altay T 2004; Qu XW 2005; Iijima H 2005; Enkhbaatar P 2009), but anti-inflammatory properties have also been described by others (Beck PL 2004). In rats, nNOS supresses iNOS expression (via a nuclear factor-kappa B (NF-

κB)/IκBα dependent mechanisms; Qu XW 2001), while inhibition of nNOS (with 7-NI) for four days) caused tissue invasion of PMNs (as assessed by intestinal myeloperoxidase enzyme activity; MPO) (Qu XW 2001). The paradoxical role of nNOS-mediated NO signaling during inflammation may result from the differences in disease severity (colitis, sepsis, cerebrovascular inflammation, etc), sample timing, and, of course, in the animal model used, i.e. rodents versus large animals).

Another possible source of NO is enzyme-independent and could be derived from tissue accumulation of nitrite during the ischemic phase (Zweier JL 1995). There are multiple pathways of NO formation from nitrite in biological systems under ischemic conditions. Nitrite is reduced to NO at the molybdenum site of the xanthine oxidase enzyme with xanthine, NADH and 2,3-dihydroxybenz-aldehyde substrates (Li H 2001). NO is also generated from nitrite reduction by the cytosolic molybdenum enzymes aldehyde oxidase. These NOS-independent NO productions from nitrite are produced primarily in tissues not in the blood and regulated by pH, oxygen tension, and nitrite, as well as by reducing substrate concentrations in the rat liver and heart (Li H 2008).

1.5. Ischemic preconditioning (IPC) in the intestine and the role of NO

Ischemic preconditioning (IPC) with brief periods of intermittent ischemia is known to be a potent intervention to reduce the consequences of IR. Its aim is to increase the ischemic tolerance of tissues. This approach was first described in the heart (Murry CE 1986), but it was later demonstrated in many organs, including the intestine (Hotter G 1996; Tamion F 2002). This multifactorial defensive cellular phenomenon involves many targets and mechanisms (including malondialdehyde (MDA), TNF-α, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1)) in the intestine, including moderation of the severity of oxidative stress and activation of endogenous cellular adaptation processes in bowel tissue (Sola A 2000; Ferencz A 2002; Ji YY 2015). The attenuating effect of IPC against early stages of inflammatory response (Camprodon RA 2014) includes down-regulation of the expression of transcription factor NF-κB (Ferencz A 2002; Wei Z 2009) with resultant cytoprotection via the inhibition of apoptotic cascade (Maulik N 1999). IPC-induced improvement of IR injury also manifests in protection of the villous microcirculation in rodents (Mallick IH 2005b) and in prevention of intestinal motility dysfunction via preservation of jejunal enteric nerve function in rats (Taha MO 2012). In other studies (performed on rats), IPC exerted marked protection of intestinal mucosa (in particular villus tips), which manifest in reduced mucosal permeability (McCallion K 2000; Maretta M 2012,) and apoptosis (Sileri P 2004) in the early reperfusion

phase (<120 min) and in enhanced IPC-induced mucosal regeneration in the later phase (Maretta M 2012).

Similarly to IR, the protective role of IPC was also demonstrated with SBTX. The protective effect of IPC is associated with altered gene expression (cell adhesion, energetic and substrate metabolism, and early response gene activation) in SBTX at early stages of reperfusion in rats (Wang S 2009), IPC-ameliorated oxidative stress (MDA, superoxide dismutase (SOD), GSH and XOR) (Sola A 2000; Ferencz A 2004) and inhibited NF-κB expression after SBTX in dogs (Ferencz A 2002), and reduced apoptotis in rats (Wang SF 2003). Furthermore, IPC has been shown to ameliorate IR-induced endothelial activation as evidenced by reduced early shedding of adhesion molecule ICAM-1 from the endothelial surface during SBTX (Camprodon RA 2014). During heterotopic SBTX in rats, the IPC-evoked release of hem-oxigenase-1 is thought to play a role in reducing systemic consequences (lactate-dehydrogenase (LDH), aspartate-aminotransferase and alanine-aminotransferase) of SBTX (3 h reperfusion; Saeki I 2011).

Some of the protective effects of IPC (e.g. LDH and morphology) were also attributed to NO-dependent mechanisms in the intestine (Hotter G 1996; Sola A 2001). A transient NO release at the onset of reperfusion could be directly detected by NO-sensitive microelectrodes in different organs (testis: Kono T 2006; heart: Takahashi SS 2003; liver: Lhuillier F 2003; kidney: Saito M 2000; urinary bladder: Saito M 1999; stomach: Wada K 1998; brain: Ohta K 1996) and in the bowel (Watanabe T 2007). Interestingly, remote IPC resulted in an enhanced NO release during both the ischemic and reperfusion phases in the heart (Kang SW 2013). With SBTX, NO donors mitigated reperfusion injury after IPC+SBTX (Sola A 2001) and reduced tissue of MDA and MPO levels at early reperfusion (Cao B 2005), whereas NOS inhibition with $N(\omega)$-nitro-L-arginine methyl ester (L-NAME) prevented the IPC-induced protection (as concerns LDH and MPO changes) (de Oca 1999) in rats. Therefore the involvement of Ca^{2+}-dependent NOS (cNOS)-derived NO in IPC-induced protection seems to be confirmed, but the potential role of nNOS is examined to a lesser extent. Although IPC did not decrease the size of the infarct area in eNOS or nNOS knockout mice (Atochin DN 2003), nNOS was found to be protective for heart IPC both *in vitro* (against necrosis, apoptosis and CK release) and *in vivo* (via mitoKATP and p38MAPK-dependent pathways, Barua A 2010; oxidative/nitrosative stress, Lu XM 2009) as the positive effect of IPC is lost after nonspecific NOS inhibition and in nNOS knock out animals (Lu XM 2009). Furthermore, the post-translational modification and

modulatory effect of IPC on nNOS activity were also demonstrated in a cerebral ischemia model (Wang M 2016).

1.6. Mast cell (MC)-driven cellular reactions during intestinal IR and SBTX – possible interplay between NO, PMNs and MCs

The mechanisms of IR injury involve not only direct tissue injury (elicited by the IR insult), but also secondary (usually also delayed) damage caused by inflammatory activation (Sheridan AM 2000). Activation of mast cells (MCs) is one of the manifestations of IR-related inflammatory reactions (Boros M 1995; Andoh A 2001). MCs are located close to the arterioles and venules of the gastrointestinal mucosa (Kunder CA 2011), and a contact similar to a synapse also exists between nerves and MCs (Stead RH 1989; Blennerhassett MG 1991). MC activation/degranulation can be brought about by both extracellular and intracellular pathways. Extracellular pathways are mediated by a series of G protein-coupled receptors (Kuehn HS 2007), e.g. endothelin-A receptors (Szalay L 2000), adenosine receptors 2A and 3 (Fozard JR 1996) and anaphylatoxin receptors C3a or C5a (Fukuoka Y 1990) on the MC surface. The intracellular pathways include MC degranulation without extrinsic signals or involvement of cell surface receptors, and these pathways are linked to ROS formation (Suzuki Y 2005). These signals typically induce phospholipase C activation and calcium mobilization, leading to MC degranulation (Fukuoka Y 1990; Murray DB 2004; Kuehn HS 2007) with a resultant release of chemicals, including histamine (Boros 1991) and chymase (Galli SJ 2005). ROS generated during the reoxygenation phase can alone trigger intracellular signal transduction pathways of MCs (Lefer DJ 2000; Matsui T 2000) and resultant MC degranulation (Becker BF 2000). Apart from preformed molecules, *de novo* synthesis of mediators with high pro-inflammatory potential are also produced by MCs, e.g. TNF-α (Bischoff SC 2009), platelet activating factor (Kurose I 1997), leukotriene B4 (Su M 1993), prostaglandin D_2 (Wingard CJ 2011) and chemoattractants, including CXCL1/CXCL2 (Katsanos GS 2008; De Filippo K 2013) and CC chemokines (Wan MX 2003). Some of the reactions are also modulated by nerve plexus-MC interactions in the gut (Tikoo S 2018). The above chemoattractants bring about PMN accumulation in target damaged tissue (Kanwar S 1994d; Strbian D 2006; De Filippo K 2013), and pro-inflammatory cytokines released during MC degranulation induce expression of adhesion molecules (selectins and integrins) (Zhang J 2011), which further lead to PMN transmigration (Ley K 2007; Woodfin A 2011; Muller WA 2011; Cerutti C 2017; Schimmel L 2017). A correlation between MC-induced increase in MPO activity and the extent of small intestinal IR injury has also been demonstrated (Andoh A 1999; Gan X

2013). Of note, contribution of MCS to the IR-induced increase in microvascular (Kubes P 1996) and mucosal permeability (Kanwar S 1994a; Szabó A 1997) has been demonstrated as well.

It appears that NO is able to inhibit MC-induced PMN rolling and adhesion in the mesenteric venules (Gaboury JP 1996; Niu XF 1996) and endogeneously produced NO attenuates intestinal IR injury by ameliorating MC degranulation in rats (Hutcheson IR 1990). Activation of mucosal or serosal MCs leads to the production of NO (Hirji NS 1999) through induction of iNOS in rodents (Bidri M 1997). Interestingly, an interaction between nerves and MCs has also been demonstrated: (1) expression of complementary receptors on MC and neighboring nerves were demonstrated, which may bring about receptor-mediated activation of intracellular signaling pathways (Schemann M 2013); (2) neurotransmitters and mediators released by neurons influence MC degranulation (Galli SJ 2008); (3) impulses generated by neurons during inflammation result in release of adenosine triphosphate, vasoactive intestinal peptide, substance P and calcitonin gene-related peptide, and these substances are known to trigger MC activation (Buhner S 2012).

2. MAIN GOALS OF THE STUDIES

The main goal of the studies was to determine the consequences of local intestinal IPC in a detailed fashion in a large animal model. Since microcirculatory reactions can affect the blood supply as well as microvascular inflammatory reactions and can critically influence the integrity of the mucosal barrier during IR injury, major emphasis was put on the effect of IPC on these changes in relation to SBTX. Thus, we directly observed the intestinal microcirculations by intravital microscopy (using conventional fluorescence IVM and OPS imaging methods) in order to assess the responses to a local IR challenge elicited by cold ischemia.

Since NO has been implicated as a trigger/mediator in the IPC mechanism and since nNOS is a dominant isoform of NOS in the gastrointestinal tract, an additional aim of our studies was to examine the possible role of nNOS in the local IPC-induced protection in the intestine with particular interest in intestinal motility function and inflammatory reactions.

- In this context, 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-NI, a specific inhibitor nNOS was administered prior to IPC. Changes in (1) macro- and microhemodynamics, as well as in (2) leukocyte accumulation, (3) MC activation, (4) intestinal motility, (5) local NO production, and (6) structural injury were assessed.

3. MATERIALS AND METHODS

The experiments were performed in 2 major studies on large animals. 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. 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. Animals

The experiments complied with the ARRIVE guidelines and the project was approved by the National Scientific Ethics Committee on Animal Experimentation (National Competent Authority) in Hungary under license numbers VI./15.1/00755/001/2005 and V./148/2013. The study was performed in compliance with Directive 609/86/EEC and 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).

3.2. Surgical preparation

In both studies, outbred dogs of either sex (body weight range: 12.2–17.3 kg) were obtained from the Animal House of the University of Szeged. The animals were deprived of food, but not water, for 24 h prior to the experiments. The animals were anesthetized intravenously with sodium pentobarbital (30 mg/kg iv). Endotracheal intubation was applied to facilitate respiration, and body temperature was maintained at 37°C with a homeothermic blanket. In aseptic techniques, polyethylene catheters were surgically inserted into the femoral artery and vein to measure blood pressure and administer fluids or drugs, respectively. All the animals received a continuous infusion of Ringer's lactate at a rate of 10 ml/kg/h during the experiments.

3.3. Experimental protocols

Study 1

In this study, 8 male and 7 female outbred dogs were randomly allocated to sham-operated (n=5; male=3, female=2) and SBTX (n=10; male=5, female=5) groups. After anesthesia and vessel cannulations (see above), a Swan-Ganz thermodilution catheter (Corodyn TD-EN, 5011-110-7Fr; Braun Melsungen AG, Melsungen, Germany) was placed into the pulmonary artery via the left femoral vein to measure cardiac output by thermodilution. Following median laparotomy, the root of the superior mesenteric artery (SMA) was

dissected free, and an ultrasonic flow probe (Transonic Systems Inc., Ithaca, NY, USA) 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 and to obtain blood samples (see later).

In animals where orthotopic SBTX was performed, the spleen was first removed after an upper transverse laparotomy and the root of the SMA and vein was dissected free. The mesentery of the small bowel was divided between ligations in the distal (ileocolic) and proximal (duodenojejunal) parts, and the intestinal wall was transected. The SMA was then cannulated with a 7-F polyethylene catheter, and the small intestine was removed from the abdominal cavity. The intestinal graft was perfused with 500 ml chilled (4°C) heparinized Ringer's lactate at a perfusion pressure of 100 cm water. The graft was stored on ice until the vessels were reanastomosed end-to-end with 6/0 running sutures (nonabsorbable polypropylene). After 60-min cold ischemia, reperfusion was induced and the macro- and microhemodynamic changes were observed hourly for 4 h after revascularization. The SBTX group (n=5) went through the above procedures only, and, in the following group, the experimental protocol was identical to that described above, except that an IPC was also performed (3 times 5-min SMA occlusion, followed by 10 min of reperfusion) 60 min prior to ischemia induction (IPC+SBTX group, n=5). The last group served as a sham-operated control, without bowel harvesting (sham-operated group, n=5). In these animals, a laparotomy and splenectomy were performed and the mesenteric vessels were cannulated for hemodynamic measurements. All manipulations were identical to those in the other, treated groups, with the exception that the bowel was not perfused and harvested. There was no significant difference between the 3 groups as concerns the duration of the surgical preparation or the cold ischemia time in SBTX and IPC+SBTX groups. In this study, macro- and microhemodynamic measurements were performed every 60 min (see later) (Figure 1).

After a stabilization period, the basal cardiovascular parameters were measured for 20 min. Control intravital microscopic measurements (functional capillary density (FCD), epithelial thickness and red blood cell velocity (RBCV); see later) were performed before perfusion (Figure 1). For technical reasons (the experimental animal needs to be transported beyond the microscope), certain baseline microcirculatory measurements (to assess PMN-endothelial interactions; see later) were not taken before perfusion. After these measurements in the IPC+SBTX group, complete SMA occlusion was performed 3 times

for 5 min, followed by 10 min of reperfusion. Sixty minutes after IPC, perfusion of the graft was performed. Then, after 60 min cold ischemia vascular anastomoses were conducted, and reperfusion was monitored for 4h. Leukocyte-endothelial cell interactions were monitored hourly by IVM during the 4-hour reperfusion phase. Tissue biopsies were also harvested from the intestine for further histological (MC degranulation) analyses (at baseline and 240th min of reperfusion) (Table 1).
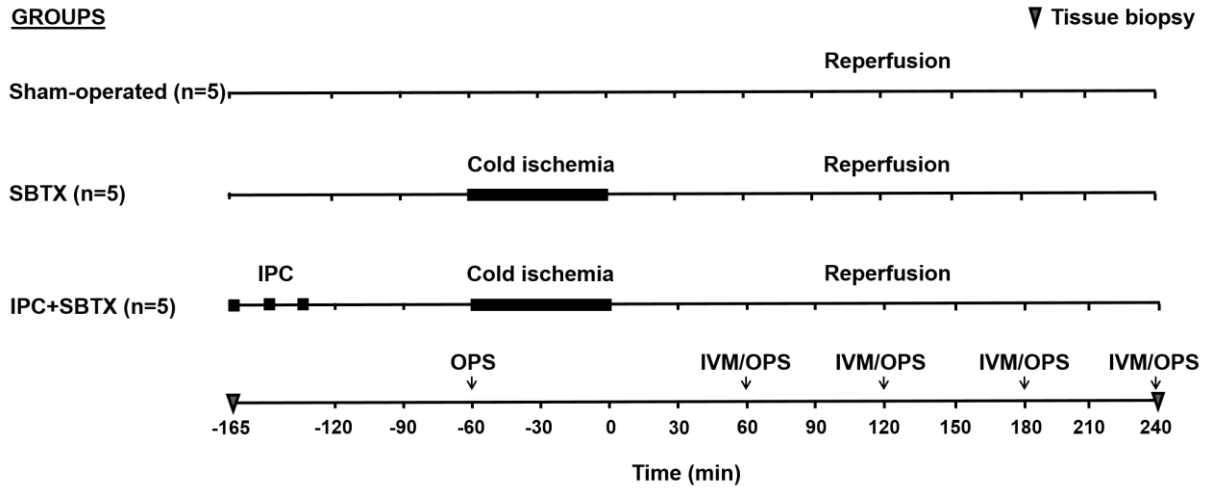

Figure 1. Experimental protocol of Study 1. Orthotopic small bowel transplantation (SBTX) was performed, and the intestinal graft underwent 60 min cold ischemia, which was followed by 240 min of reperfusion (SBTX group, n=5). In another group, an identical experimental protocol was performed except that cold ischemia was preceded 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 a 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, epithelial thickness and red blood cell velocity) and enterocyte injury in the intestinal villi before harvesting and hourly during the reperfusion phase after SBTX. Tissue biopsies from the affected bowel area (marked by an arrowhead) were also taken for histological assessments of mast cell degranulation.

Study 2

In the second study, 10 male and 9 female outbred dogs were randomly allocated to IR (n=7; male=4, female=3) and IPC (n=12; male=6, female=6) groups (Figure 2). 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 to 7-NI-treated or vehicle groups and were treated with the selective nNOS inhibitor 7-NI (5 mg/kg; n=6; male=3, female=3) or saline vehicle (n=6; male=3, female=3) in an iv bolus 15 min prior to IPC. IPC was induced in 2 groups by clamping the SMA using 3 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, USA) was dissolved in a mixture of 75% DMSO/saline and stored at –20°C; the aliquots were diluted in 3 ml of 0.9% saline before administration. In the IR group, the animals were treated with the saline vehicle at a matching time point.

Blood samples were collected from the mesenteric vein for later determination of plasma nitrate/nitrite (NO_x) levels at different time points (see at Figure 2). Tissue biopsies were also harvested from the intestine for further biochemical/histological (e.g. MPO, NOS activity and MC degranulation) analyses (at baseline as well as at the 15th and 120th min of reperfusion) (Table 1).
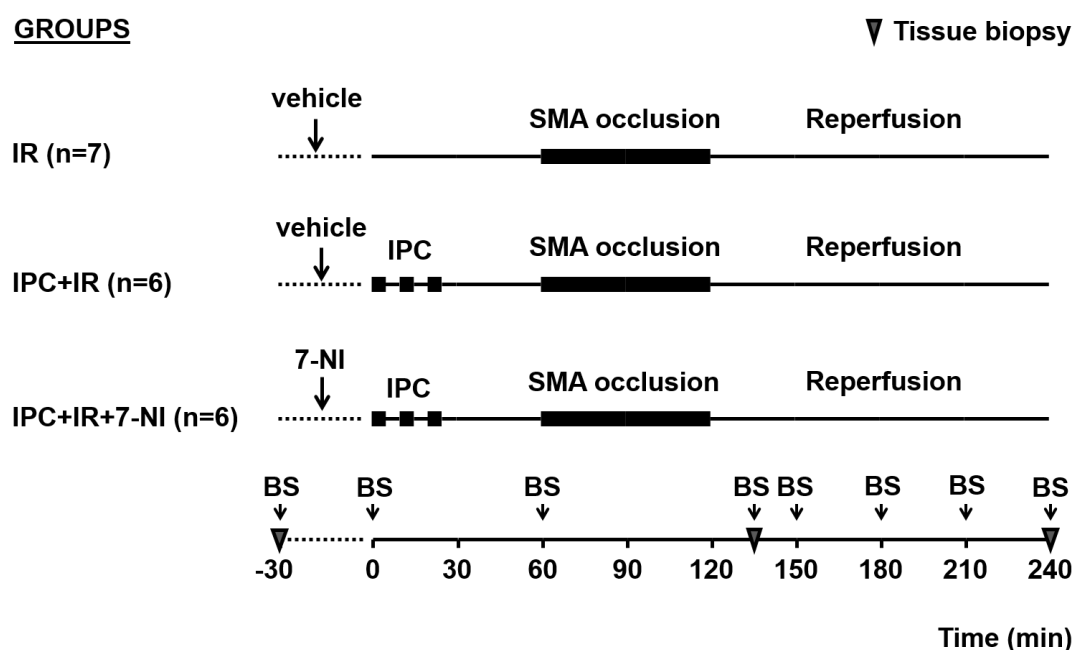
Figure 2. Experimental protocol of Study 2. In the first group, 60 min of ischemia was induced by occluding the superior mesenteric artery (SMA), which was followed by 120 min of reperfusion (IR). In the two other groups, IR was preceded by ischemic preconditioning (IPC), which was elicited by inducing 3 cycles of 5-min ischemia/5-min reperfusion periods in the presence and absence of a bolus injection of 7-nitroindazole (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 levels and nitric oxide synthase and myeloperoxidase activities, as well as to assess mast cell 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)	+	+

3.4. Hemodynamic and blood gas measurements; calculation of intramucosal pH (pHi)

In both studies, the peripheral arterial and mesenteric venous pressures as well as SMA flow (using Statham P23 Db transducers) were registered with a computerized data-acquisition system (Haemosys 1.17 Experimetria Ltd., Budapest, Hungary). In Study 1, a Swan-Ganz thermodilution catheter (Corodyn TD-EN, 5011-110-7Fr; Braun Melsungen AG, Melsungen, Germany) was also placed into the pulmonary artery via the left femoral vein to measure cardiac output by thermodilution. In Study 2 a silastic balloon catheter (TGS Tonomitor, Tonometrics Inc., Worcester, MA, USA) was introduced through a small enterotomy into the intestinal lumen. Arterial blood gases and intramucosal pCO_2 were measured with a blood gas analyzer (AVL, Graz, Austria). Intramucosal pH (pHi) was calculated by using the modified Henderson–Hasselbach formula with a correction factor for 30 min of equilibration.

3.5. Intravital fluorescence videomicroscopic (IVM) examinations of PMN-endothelial interactions

In the first Study, the mesenteric microcirculation was analyzed using an epi-illumination technique. An ileal segment with intact neurovascular connections was gently placed on a specially designed stage. During the entire *in vivo* microscopic procedure, the tissue was constantly bathed in 37°C Ringer's lactate to avoid drying and exposure to ambient air. Leukocyte-endothelial cell interactions in the postcapillary venules of the mesentery were monitored with a high-resolution Zeiss Axiotech Vario 100 HD fluorescent intravital microscope (100 W HBO mercury lamp, Acroplan 20 X water immersion objective, Ex: 495 nm, Em: 515 nm). The microscopic images were recorded by a charge-coupled device video camera (AVT HORN-BC 12) attached to an S-VHS video recorder (Panasonic AG-MD 830) and a personal computer. Leukocytes were stained *in vivo* by means of rhodamine-6G (Mw 479, Sigma, St. Louis, MO, USA; 0.2%, 0.1 ml iv). Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images (Pictron Ltd., Budapest, Hungary) within 5 postcapillary venules per animal, including the observation of nonadherent, adherent and rolling leukocytes. Adherent leukocytes (stickers) were defined in each vessel segment as cells that did not move or detach from the endothelial lining within an observation period of 30 seconds and are given as the number of cells per square millimeter of endothelial surface, calculated from the diameter and length of the vessel segment observed, assuming cylindrical geometry. Rolling leukocytes were defined as cells moving at a velocity of less than 2/5 of that of the erythrocytes in the centerline of the microvessel and are given as a percentage of the nonadherent leukocytes passing through the observed vessel segment within 30 second.

3.6. Orthogonal polarization spectral (OPS) imaging technique for assessment of microvascular velocity and morphological changes of the intestine

In the first study, an intravital OPS imaging technique (Cytoscan A/R, Cytometrics, PA, USA) was used to monitor microvascular perfusion changes in the intestinal villi before harvesting and during the reperfusion phase after transplantation. The OPS imaging technique utilizes reflected polarized light at 548 nm, which is the isosbestic point of oxy- and deoxyhemoglobin. Since polarization is preserved in reflection, only photons scattered from relatively deep inside the tissue contribute to the images. In this way, a virtual light source is created in the tissues so that the vessels appear black. The method is

suitable for visualizing all hemoglobin-containing structures, without the use of a fluorescent dye. A 10x objective was introduced into the intestinal lumen through a small enterotomy on the antimesenteric side of the bowel. The objective was connected to a light source with flexible cables; thus, this intestinal segment was not exteriorized during the study. Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. The FCD (length of perfused nutritive capillaries per observation area (centimeter –1), the RBCV (millimeters/second) in the central arterioles and venules of the villi and the thickness of the epithelial cell layer covering the intestinal villi (micrometers) were determined in 5 separate fields by means of a computer-assisted image analysis system (IVM Pictron®). All data were expressed as the means of 5 measurements at each time point.

3.7. Intestinal motility measurements

In Study 2, a modified strain gauge transducer technique was applied to monitor the small bowel motility, and the motility index was calculated to estimate the neurogenic integrity of the intestine (Palásthy Z 2006). Briefly, two strain gauge transducers (FSG-02 type; size: 6x15 mm; Experimetria Ltd., Budapest, Hungary) were sutured to the circular muscle layer of an ileum segment in a parallel position using 5/0 silk suture material (Braun-Dexon, Melsungen, Germany). The strain gauge transducer wire was connected to an SG-M bridge amplifier, and the signals were continuously recorded with a computerized data-acquisition system (Haemosys 1.17; Experimetria Ltd., Budapest, Hungary). Signal analysis was performed offline, and the bowel motility index was determined by calculating the area under the motility curve as a function of time (Huge A 1998).

3.8. Plasma nitrate/nitrite (NOx) level measurements

In Study 2, plasma NO_x concentrations were determined from samples taken at baseline (t= –30 min), before ischemia as well as at the 15th, 60th and 120th min of reperfusion by means of the Griess reaction. Blood samples were centrifuged at 10 000 g for 15 min at 4°C, and the plasma samples were stored at –20°C for a maximum of 2 months. The plasma was mixed with b-nicotinamide adenine dinucleotide phosphate, flavin adenine dinucleotide and nitrate reductase (Sigma-Aldrich Co., St. Louis, MO, USA) and incubated for 30 min at 37°C. Following the enzymatic reduction of nitrate to nitrite, the Griess reagent was added to the mixture and incubated for an additional 10 min at room temperature. The absorbance of the azo compound was measured spectrophotometrically at a wavelength of

540 nm, and the total NO_x concentration (mmol/l) was determined using a standard calibration curve of $NaNO_2$ and $NaNO_3$ (Sigma-Aldrich Co., St. Louis, MO, USA).

3.9. Measurement of nitric oxide synthase (NOS) enzyme activity

In Study 2, a specific method to determine NOS activity based on the enzymatic conversion of ^{3}[H]-L-arginine to ^{3}[H]-L-citrulline was described earlier (Palásthy Z 2006). Briefly, intestinal tissue biopsies were homogenized on ice in a phosphate buffer (pH 7.4; 50 mM Tris-HCl, 0.1 mM EDTA, 0.5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml soybean trypsin inhibitor, 10μg/ml leupeptin), and then the homogenate was centrifuged at 24000 g for 20 min at 4°C. The supernatant was transferred to centrifugal concentrator tubes (Amicon Centricon-100; 100000 MW cut-off ultrafilter, Merck KGaA, Darmstadt, Germany), and samples were centrifuged at 900 g for 150 min at 4°C. After the second centrifugation, the concentrated supernatant was washed out of the ultrafilter with 250 μl homogenizing buffer. Samples were incubated for 5 min with a cation-exchange resin (Dowex AG 50W-X8, Na^+ form, Dow Chemical Company, Midland, MI, USA) to deplete endogenously produced L-arginine. The resin was removed by centrifugation at 1500 g for 10 min and a supernatant-containing enzyme was used for a NOS activity assay. cNOS activity was assessed in the presence of a 50 μl enzyme extract and a 100 μl reaction mixture (pH 7.4, containing 50 mM Tris-HCl buffer, 1 mM NADPH, 10 μM tetrahydrobiopterin, 1.5 mM $CaCl_2$, 100 U/ml calmodulin and 0.5 μCi [^{3}H] L-arginine (Amersham BioSciences UK Ltd,, Amersham, UK) specific activity 63 Ci/mmol) after 60 min of incubation at 37°C. The enzyme reaction was terminated by adding 2 mM EGTA and 2 mM EDTA containing an ice-cold HEPES buffer (pH 5.5, in 1 ml volume of buffer). NOS-independent ^{3}[H]-L-citrulline formation was assessed in the presence of a non-selective NOS inhibitor, N-ω-nitro-L-arginine (3.2 mM, Sigma Chemical Co., St. Louis, MO, USA). Ca-calmodulin was omitted in determining Ca^{2+}-independent NOS activity (iNOS), while EGTA (8 mM) was added to the reaction mixture. A 1-ml reaction mixture was applied to the Dowex cation-exchange resin (AG 50W-X8, Na^+ form) and eluted with 2 ml dH_2O. The eluted [^{3}H] L-citrulline activity was determined with a scintillation counter (Tri-Carb Liquid Scintillation Analyzer 2100TR/2300TR; Packard Instrument Co., Meriden, CT, USA). Total protein concentrations in samples were determined using the Lowry method.

3.10. Tissue myeloperoxidase enzyme activity (MPO) measurements

In Study 2, MPO, a marker of neutrophil granulocyte infiltration, was measured from mucosal biopsies (Kuebler WM 1996). Briefly, the tissue was homogenized with Tris-HCl buffer (0.1 M, pH 7.4) containing 0.1 mM polymethylsulfonyl fluoride to block tissue proteases and then centrifuged at 4°C for 20 min at 5000 g. The MPO activities of the samples were measured at a 450 nm wavelength (UV-1601 spectrophotometer, Shimadzu, Japan), and values were normalized to protein content.

3.11. Determination of MC degranulation

In both studies, ileal biopsies were placed into an ice-cold Carnoy's fixative, embedded in paraffin, sectioned (6 μm) and stained with Alcian blue and Safranin O. Histological analysis was performed in coded sections by one investigator at 40x 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 (iMC) and degranulated (dMC) mast cells: MC% = dMC / (dMC+iMC) (Gera L 2007).

3.12. Determination of morphological changes in the structure of the small intestine

In the first Study, the thickness of the epithelial cell layer covering the intestinal villi (micrometers) were determined using the OPS method in 5 separate fields by means of a computer-assisted image analysis system (IVM Pictron Ltd, Hungary)(see above).

In Study 2, analysis of morphological 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 (%) as well as (2) villus height – crypt height ratio (%); these were determined for each villus of the current slide (at 3–5 high power fields at 4x 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 10x magnification) (Chiu CJ 1970).

3.13. 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. Median values, and 75th and 25th percentiles are illustrated in figures. Results were considered significant at $P < 0.05$ value.

4. RESULTS

Study 1

4.1. Macrohemodynamic effects of IPC during SBTX

MAP decreased significantly during the reperfusion phase, with no differences in this parameter between the SBTX and IPC+SBTX groups (data not shown). 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 (Figure 3). 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 (Figure 4).
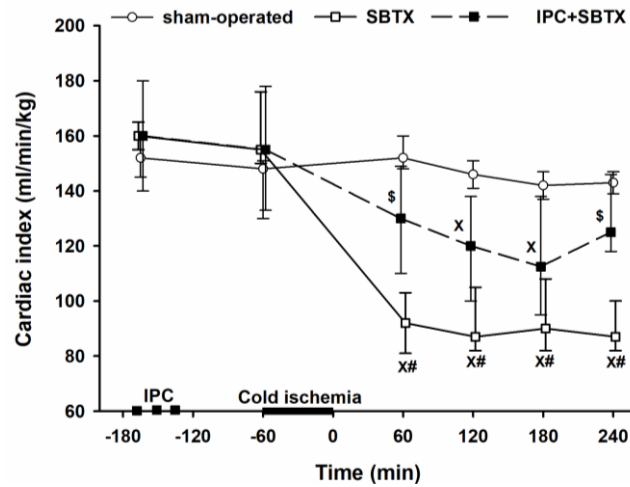

Figure 3. Changes in cardiac index in animals subjected to small bowel transplantation (SBTX) in the presence and absence of ischemic preconditioning (IPC). Measurements were performed at baseline and hourly during a 240 min reperfusion period after 60 min of cold ischemia and at matching time points in sham-operated animals (not subjected to ischemia). Median values as well as 25th and 75th percentiles are indicated. 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 $P < 0.05$ versus baseline values, # $P < 0.05$ versus sham-operated group, \$ $P < 0.05$ between SBTX and IPC + SBTX groups.
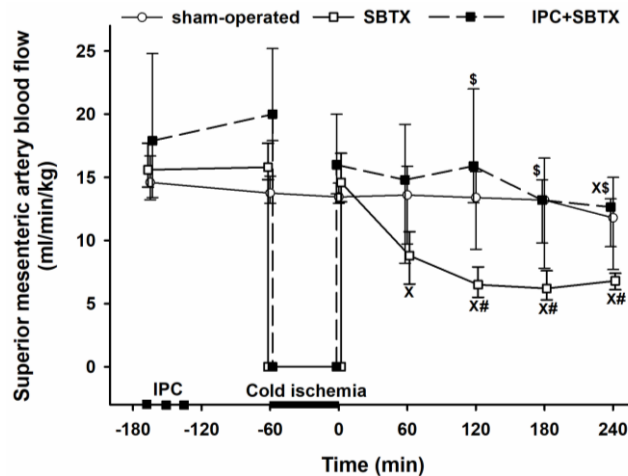
Figure 4. Changes in superior mesenteric artery flow in animals subjected to small bowel transplantation (SBTX) in the presence and absence of ischemic preconditioning (IPC). Measurements were performed at baseline and hourly during a 240 min reperfusion period after 60 min of cold ischemia and at matching time points in sham-operated animals (not subjected to ischemia). Median values as well as 25th and 75th percentiles are indicated. 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 $P < 0.05$ versus baseline values, # $P < 0.05$ versus sham-operated group, $ $P < 0.05$ between SBTX and SBTX + IPC groups.

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 (Figure 5) and the number of firmly adhered white blood cells as well (Figure 6).
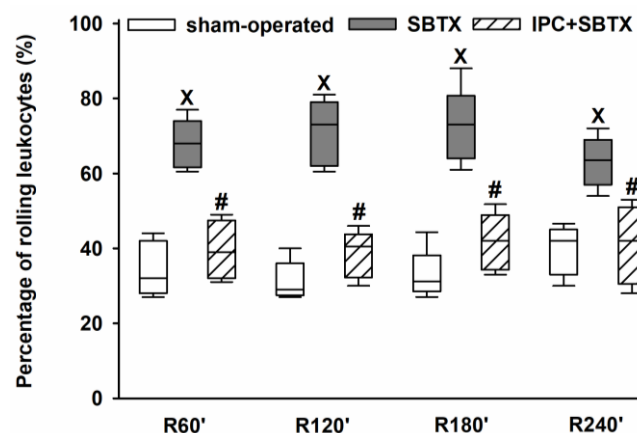
Figure 5. Changes in leukocyte rolling in ileal postcapillary venules of animals subjected to small bowel transplantation (SBTX) in the presence and absence of ischemic preconditioning (IPC). Measurements were performed hourly during a 240 min reperfusion period after 60 min of cold ischemia and at matching time points in sham-operated animals (not subjected to ischemia). 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 $P < 0.05$ within the groups versus R60' values, # $P < 0.05$ versus sham-operated group.

Figure 6. Changes in number of adherent leukocytes in ileal postcapillary venules of animals subjected to small bowel transplantation (SBTX) in the presence and absence of ischemic preconditioning (IPC). Measurements were performed hourly during a 240 min reperfusion period after 60 min of cold ischemia and at matching time points in sham-operated animals (not subjected to ischemia). 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 $P < 0.05$ within the groups versus R60' values, # $P < 0.05$ versus sham-operated group.
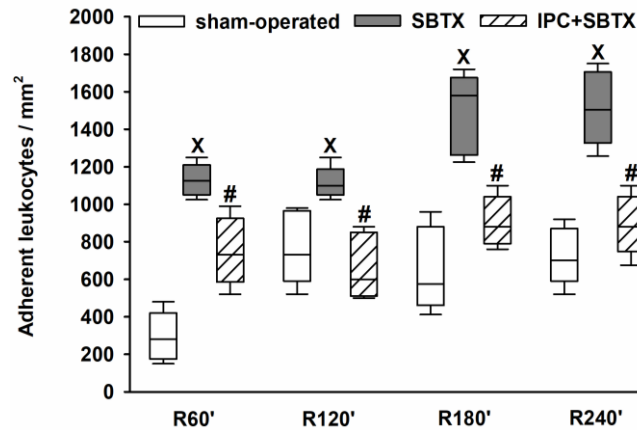
4.3. Microcirculatory effects of IPC during SBTX

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 (Figure 7).
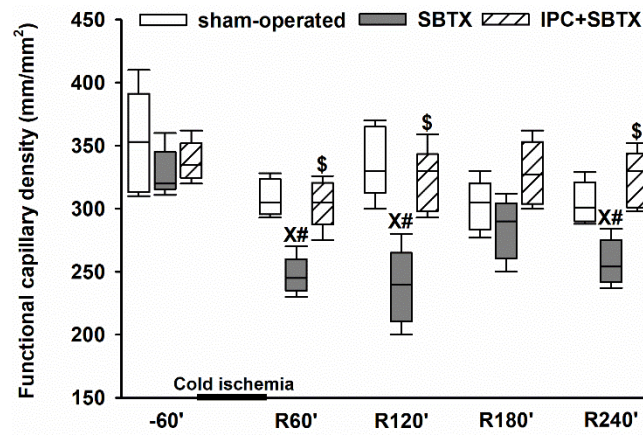
Figure 7. Changes in functional capillary density in capillaries of the ileal longitudinal muscle layer in animals subjected to small bowel transplantation (SBTX) in the presence and absence of ischemic preconditioning (IPC). Measurements were performed at t= –60 min and hourly during a 240 min reperfusion period after 60 min of cold ischemia and at matching time points in sham-operated animals (not subjected to ischemia). Here, plots demonstrate the median (horizontal line in the box), 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 $P < 0.05$ within the groups versus R-60' values, # $P < 0.05$ versus sham-operated group, $ $P < 0.05$ between SBTX and SBTX + IPC groups.

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 (Figure 8).
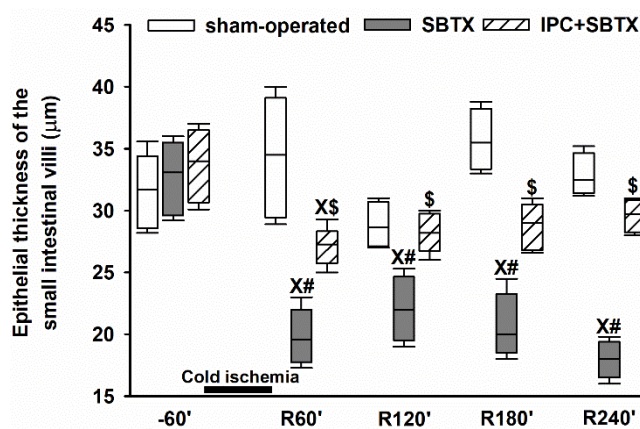

Figure 8. Changes in epithelial thickness of the small intestinal villi in animals subjected to small bowel transplantation (SBTX) in the presence and absence of ischemic preconditioning (IPC). Measurements were performed at t= –60 min, and hourly during a 240 min reperfusion period after 60 min of cold ischemia and at matching time points in sham-operated animals (not subjected to

ischemia). Here, plots demonstrate the median (horizontal line in the box), 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 $P < 0.05$ within the groups versus R-60' values, # $P < 0.05$ versus sham-operated group, $ $P < 0.05$ between SBTX and SBTX + IPC groups.

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 (Figure 9). 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.
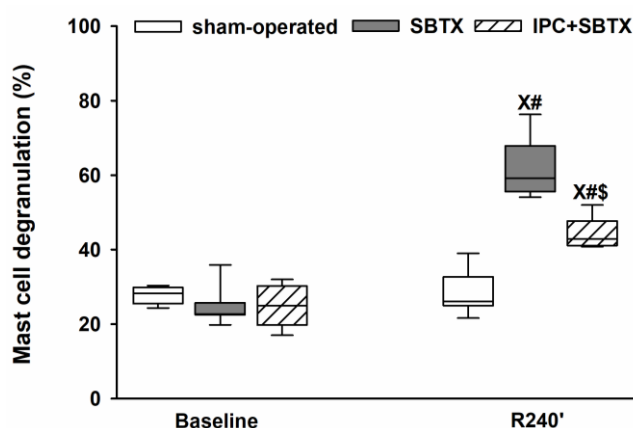

Figure 9. Changes in mast cell degranulation in ileal samples from animals subjected to small bowel transplantation (SBTX) in the presence and absence of ischemic preconditioning (IPC). Samples were taken at baseline and at 240 min of reperfusion after a 60 min of cold ischemia and at matching time points in sham-operated animals (not subjected to ischemia). Here, plots demonstrate the median (horizontal line in the box), 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 $P < 0.05$ within the groups versus baseline values, # $P < 0.05$ versus sham-operated group, $ $P < 0.05$ between SBTX and SBTX + IPC groups.

Study 2

4.6. Effects of 7-nitroindazole (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 (Figure 10). Ischemia caused an abrupt decrease (due to zero flow during occlusion in the affected intestinal area) and early reperfusion (as measured at the 5th 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.
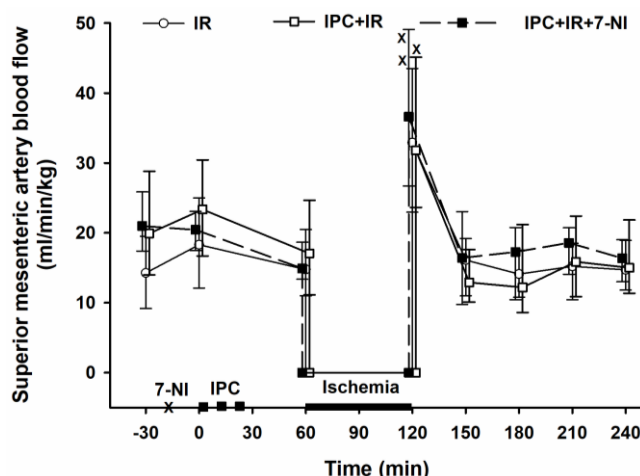

Figure 10. Time course of changes in superior mesenteric artery flow during baseline and during 60 min of SMA occlusion, followed by 120 min of reperfusion (IR group). Further groups of animals were also subjected to ischemic preconditioning (IPC) in the presence and absence of 7-nitroindazole (7-NI) treatment (IPC+IR+7NI 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 $P < 0.05$ *vs* baseline.

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 (Figure 11). 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 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.
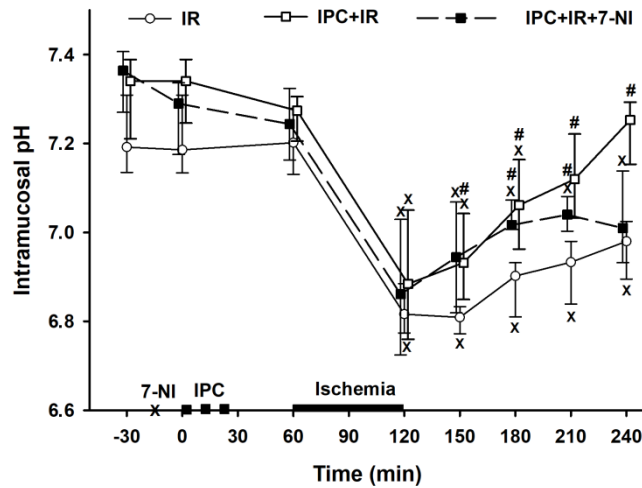
Figure 11. Time course of changes in intramucosal pH during baseline and during 60 min of SMA occlusion followed by 120 min of reperfusion (IR group). Further groups of animals were also subjected to ischemic preconditioning (IPC) in the presence and absence of 7-nitroindazole (7-NI) treatment (IPC+IR+7NI 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 *P* < 0.05 *vs* baseline, # *P* < 0.05 *vs* IR.

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 (Figure 12); 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 60th 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.
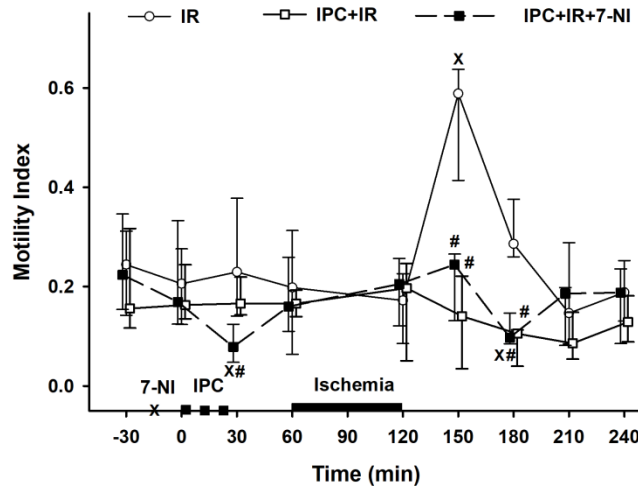
Figure 12. Time course of changes in motility index during baseline and during 60 min of SMA occlusion, followed by 120 min of reperfusion (IR group). Further groups of animals were also subjected to ischemic preconditioning (IPC) in the presence and absence of 7-nitroindazole (7-NI) treatment (IPC+IR+7NI 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 $P < 0.05$ vs baseline, # $P < 0.05$ vs IR.

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 (Figure 13). 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 (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.
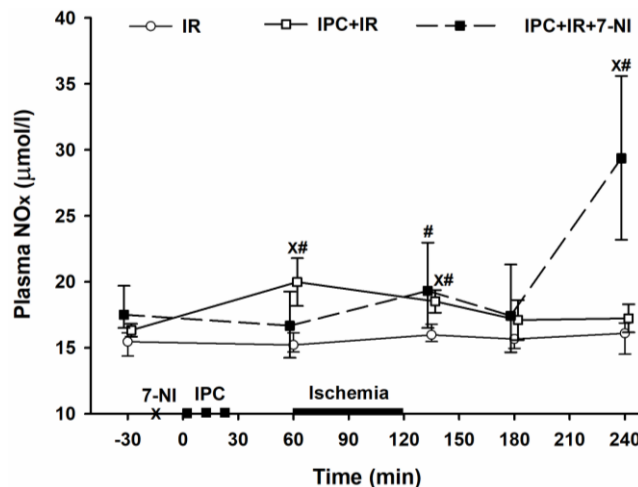

Figure 13. Time course of changes in plasma nitrate/nitrite levels (NO$_x$) during baseline and during 120 min of reperfusion after a 60-min of SMA occlusion (IR group). Further groups of animals were also subjected to ischemic preconditioning (IPC) in the presence and absence of 7-

34

nitroindazole (7-NI) treatment (IPC+IR+7NI 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 $P < 0.05$ *vs* baseline, # $P < 0.05$ *vs* IR.

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 (Figure 14A–B). As for Ca^{2+}-independent NOS (iNOS) activity, no statistically significant changes were detected throughout the entire experimental period in any of the groups (Figure 14B). 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 15th min of reperfusion, but this increase was not present in the 7-NI-treated group.
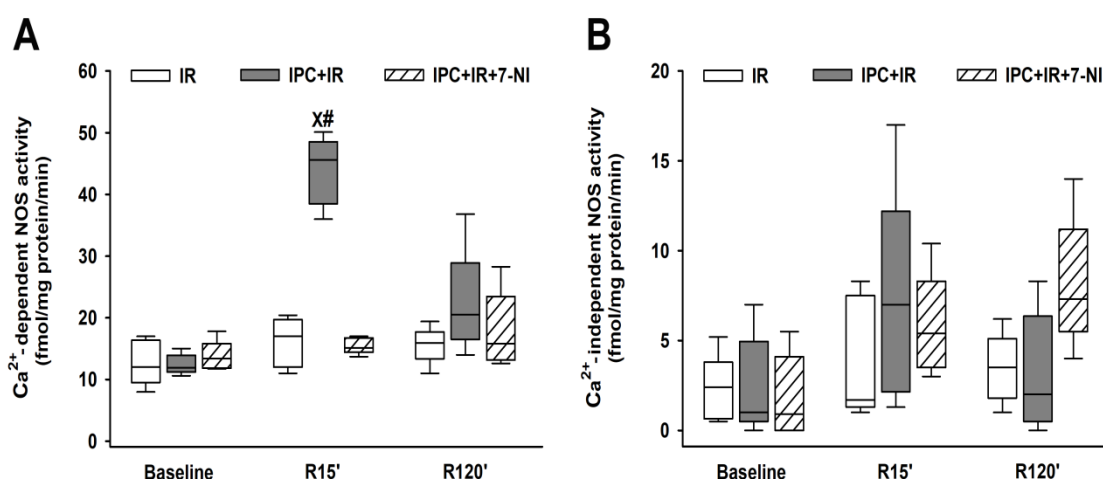
Figure 14. Changes in constitutive NO synthase (Ca^{2+}-dependent NOS, Panel A) and in inducible NO synthase (Ca^{2+}-independent NOS, Panel B) activities in the affected ileal segment during baseline, and at 15 min and 120 min of reperfusion (IR). Further groups of animals were also subjected to ischemic preconditioning (IPC) in the presence and absence of 7-nitroindazole (7-NI) treatment (IPC+IR+7NI 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 $P < 0.05$ *vs* baseline, # $P < 0.05$ *vs* IR.

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 (Figure 15), 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 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.
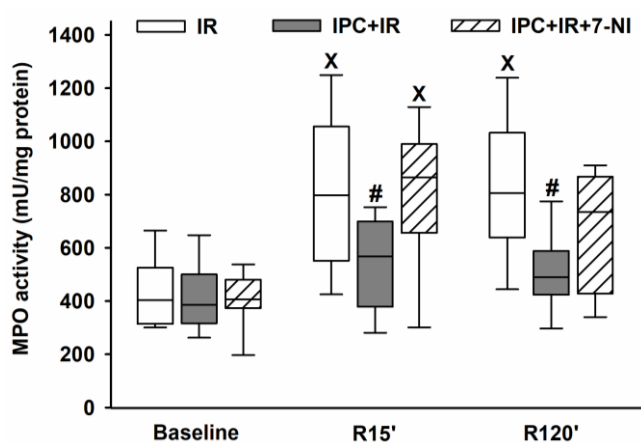
Figure 15. Changes in leukocyte accumulation (myeloperoxidase activity, MPO) in the affected ileal segment during baseline, at 15 min and 120 min of reperfusion (IR). Further groups of animals were also subjected to ischemic preconditioning (IPC) in the presence and absence of 7-nitroindazole (7-NI) treatment (IPC+IR+7NI 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 $P < 0.05$ *vs* baseline, # $P < 0.05$ *vs* IR.

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 (Figures 16A–D, 17A–B and 18A).

Fifteen minutes of reperfusion after a 60-min SMA occlusion caused a significant increase in MC degranulation (Figures 16A and 17C–D) and in mucosal injury (as evidenced by loss of enterocyte integrity and subepithelial changes) (see details in legend for Figures 17–18) and a reduction in mucosal thickness (Figure 16C–D) 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 (Figures 16 and 18C).
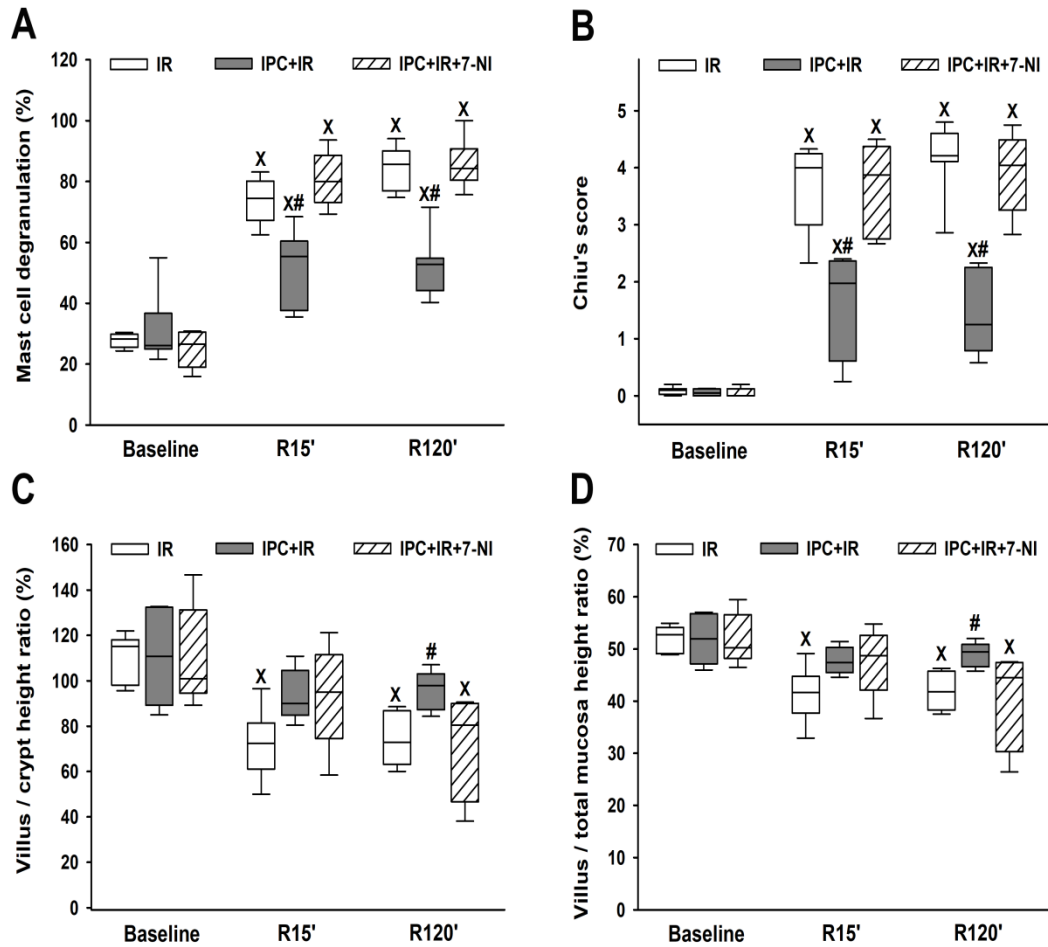
Figure 16. Changes in mast cell degranulation (Panel A), mucosal histological injury according to Chiu's grade (Panel B) and mucosal height (as expressed as villus/crypt height ratio, Panel C, and villus/total mucosa height ratio, Panel D) in samples taken from the affected ileal segment during baseline, at 15 min and 120 min of reperfusion (IR). Further groups of animals were also subjected to ischemic preconditioning (IPC) in the presence and absence of 7-nitroindazole (7-NI) treatment (IPC+IR+7NI 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 $P < 0.05$ *vs* baseline, # $P < 0.05$ *vs* IR.

Figure 17. Representative micrographs showing mucosal mast cells stained with Alcian blue and Safranin O at lower (Panels A and C; bar denotes 200 µm) and higher magnifications (Panels C and D) (bar denotes 50 µm). Please note intact intracellular granules (Panel B) and a loss of intracellular granules and stained material dispersed diffusely and extracellularly as signs of mast cell degranulation at the 120th min of reperfusion in the ischemia/reperfusion group (Panel D).
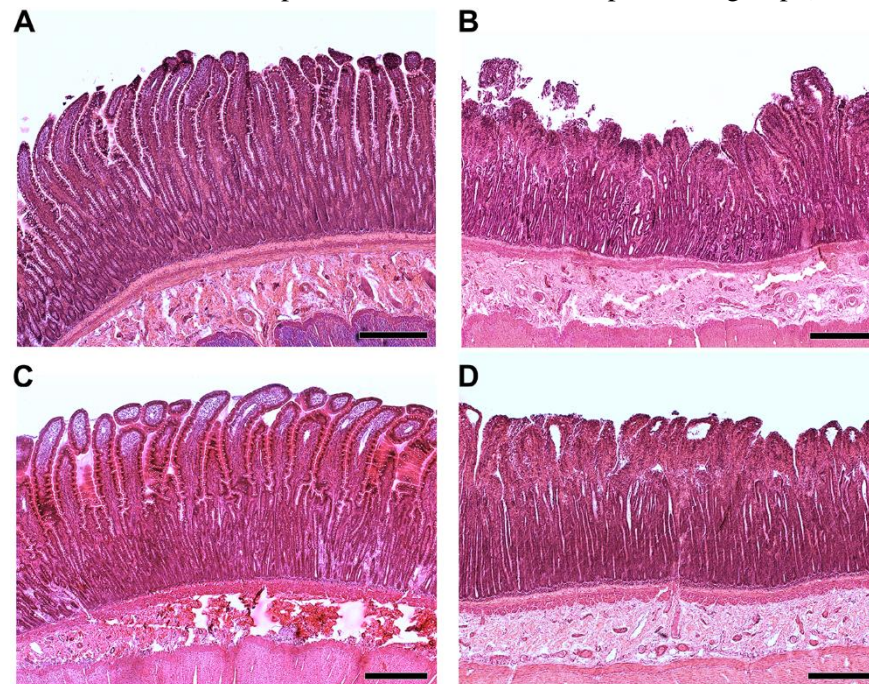
Figure 18. Representative micrographs of the ileal mucosa stained with hematoxylin and eosin staining at baseline (Panel A) and at 120 min of reperfusion in the IR group (Panel B), the IPC+IR group (Panel C) and the IPC+IR+7NI group (Panel D). Panels B and D demonstrate massive epithelial lifting down 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 µm in all figures.

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" (Haglund U 1993) 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 (Wolfárd A 2002). 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 (Gonzalez AP 1994; Wolfárd A 2002; Lautenschläger I 2018). 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 (see Gonzalez AP 1994; Cicalese L 1996; Wolfárd A 2002; Zhang XQ 2005), 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 (Carmody IC 2004). In response to warm IR, increased MPO values were observed as early as the 15th min of reperfusion in our model, but this phenomenon was observed similarly early by others after 6–24 hrs of cold ischemia in rats (Massberg S 1998; Cao B 2005). Nonetheless, the duration of cold ischemia seems to influence the extent of adhesion molecule-dependent enhancement of PMN-endothelial interactions (Preissler G 1998; Massberg S 1998; Schramm R 2007). With SBTX, IPC was shown to provide similar

prevention of tissue infiltration of PMNs (de Oca 1999). Although we did not examine it here, hypoxia-induced factor-1, a suspected mediator mechanism of IPC, is influenced by a PMN respiratory burst, which also counteracts with epithelial integrity (Karhausen J 2004; Colgan SP 2015). Furthermore, PMN transmigration during acute mucosal inflammation causes a transient increase in epithelial permeability through a protein kinase A-dependent mechanism (Haskó G 2008; Colgan SP 2012, 2015). 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) (de Groot H 2007).

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 (Ballinger WF 1962). 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 (Takayama I 2000; Matsuura T 2007), while slow wave frequency recovers much later (within 18 months) (Quigley EM 1998). It has been suggested that intestinal dysmotility leads to bacterial overgrowth due to the stasis of bowel contents in the long run (Nieuwenhuijs VB 1998). In other studies, the motility index decreased at the proximal colon after extrinsic denervation (Morita H 2012) and exogenous NO caused a dose-dependent inhibition of spontaneous and basal contractile activity in intestinal muscle strips (Zyromski NJ 2005). 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 (Boros M 1995), also modulating permeability chances both in dogs (Szabó A 1997) and rats (Andoh A 1999). MCs facilitate mesenteric afferent signaling during acute intestinal ischemia as well (Jiang W 2011). MC stabilizers (Na-cromoglycate and ketotifen) reduce bowel IR injury, e.g. Chiu's score (Hei ZQ 2007), improve skin flap survival, also influencing MPO (Cordeiro PG 2000) in rats, and ameliorate PMN-

endothelial interactions in the feline and rat bowel (Kanwar S 1994d; Kalia N 2005). MC degranulation appears to be free radical-dependent in the rat intestine, since SOD and catalase reduce this reaction (Kanwar S 1994c), but the role of endothelin also seems to be involved in this process (Szalay L 2000). MC stabilization likewise attenuates intestinal IR injury by down-regulating endothelin-1 and MPO levels in mice (Gan X 2013). MC-derived mediators were shown to be involved in the L-NAME-induced enhancement of epithelial permeability seen in the rat small intestine *in vivo* (Kanwar S 1994a). Although the results are debated, degranulation of connective tissue-type MCs also seems to be mediated by NO (van Overveld FJ 1993; Iikura M 1998). Interestingly, the positive effect of IPC was dependent on MC-dependent mediator release in the small bowel (Xing D 2014) and in the heart (Parikh V 1998) in rats. MC stabilization has been shown to increase survival in rats subjected to intestinal IR (Kalia N 2005).

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 (Ehara T 2003) and at orthotopic liver transplantation (Yang M 2014). Through the secretion of both pro- and antiinflammatory mediators, MCs also influence the course and outcome in lung transplantation (Jungraithmayr W 2015). Another aspect is the activatory effect of MCs on dentritic cells, which may have implications in transplantation biology (Goldstein DR 2005; Land WG 2006, 2007). In our studies, a similar degree of MC degranulation were 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 (Iwanami K 1998), and the extent of PMN transmigration show a good correlation with the overall severity of intestinal histological injury (Nusrat A 1997; Nash S 1987; Ginzberg HH 2001). 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) (Rauen U 2004). A similar reaction was

demonstrated in the rat liver (Ikeda T 1992; Huet PM 2004), but examination of the reactions of the cold IR-induced apoptosis did not represent an aim of the present studies.

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 neuronal nitric oxide synthase 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 (Hotter G 1996; Ferencz A 2002; Sileri P 2004; McCallion K 2000; Yang B 2018), 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 (Pontell L 2011). 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 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 (McCallion K 2000; Maretta M 2012) and apoptosis (Sileri P 2004) in the early reperfusion phase (<120 min) and in enhanced IPC-induced mucosal regeneration in the later phase (Maretta M 2012). The mechanisms of protection provided by IPC in the intestine were strongly linked to oxidative stress (MDA and TNF-α release: Ferencz A 2002; endothelial activation and

adhesion molecule release: Camprodon RA 2014 and Ji YY 2015) but were also attributed to NO-dependent mechanisms (Hotter G 1996; Sola A 2001). A transient peak in NO release was detected during the early postischemic phase in the rat intestine (Hotter G 1996), and this phenomenon was also observed if IR was preceded by IPC (Watanabe T 2007).

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 (Lai CH 2017) even as early as 5 min of reperfusion *in vitro* (in Guinea pigs) (Giaroni C 2013) or after 3 h of reperfusion *in vivo* in the rat jejunum (Lai CH 2017). 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 (15th 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 (Zweier JL 1995) 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, Rivera LR 2012). The same group found that nitrosative stress takes place mostly in a later phase of reperfusion in the enteric nerve system (Rivera LR 2011b) and in the muscle layer, which is obviously preceded by damage to the mucosa (Pontell L 2011). Furthermore, genetic ablation of the nNOS gene worsens the IR-induced bowel injury (inflammatory infiltration and motility) (Rivera LR 2012). So far, the role of nNOS in IPC has been examined to a lesser extent. Despite the previously observed debates (Altay T 2004; Beck PL 2004; Qu XW 2005; Iijima H 2005), 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*) (Barua A 2010). 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 (Lu XM 2009). Furthermore, the post-translational modification and modulatory effect of IPC on nNOS activity was also demonstrated in a cerebral ischemia model (Wang M 2016). 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 protective role of nNOS against intestinal reperfusion injury (with respect to bowel muscle contractility and PMN accumulation) (Rivera LR 2012).

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 (Calcina F 2005; Rivera LR 2011b; Taha MO 2012; Palombit K 2013). 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) (Castro M 2012) via sustained smooth muscle hyperpolarization; this causes inhibition of spontaneous motility (Gil V 2010). On the other hand, nonspecific inhibition of nNOS reduced gastrointestinal motility not only in our study, but also elsewhere (Calcina F 2005; Filpa V 2017), and we found a similar reducing effect of 7-NI on the colon motility in a subacute bowel obstruction model as well (Palásthy Z 2006). 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); it could be reserved by L-NAME and an NMDA receptor antagonism (Calcina F 2005). 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 (Rivera LR 2012), while reduced intestinal transit time (i.e. increased motility) was observed under similar circumstances after nNOS inhibition (Filpa V 2017). 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 (Moore-Olufemi SD 2005). 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 (de Oca J 1999; Xing D 2014). 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 (de Oca J 1999; Filpa V 2017). 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) (de Oca J 1999; Unal S 2003; Filpa V 2017), and this effect was also present at 6 hrs of reperfusion (Moore-Olufemi SD 2005). The nNOS dependency of the latter reaction was also proven as inhibition of nNOS (or KO) (Rivera LR 2012) reversed this protection. In a cremaster flap model, 7-NI did not influence the effect of IPC on PMN-endothelial interactions (4h/2h IR) (Sanz MJ 2001). 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 (Xing D 2014). 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) (Jansson L 2005), 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 (see Study 1; Mallick IH 2005a, 2005b). These reactions were also explained with an IPC-induced reduction of intestinal oxidative stress (Ji YY 2015). 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 NO synthase 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.

7. ACKNOWLEDGMENTS

I am grateful to Mihály Boros M.D., Ph.D., D.Sc., for providing me with the opportunity to carry out my research and for initiating my scientific career in the Institute of Surgical Research under his valuable scientific guidance.

I am indebted to my two supervisors, Andrea Szabó M.D., Ph.D., and József Kaszaki Ph.D., who have helped me acquire the basic experimental skills and granted me unlimited daily assistance in conducting studies and contributing to publications as a first author.

I am also grateful to Gábor Bogáts M.D., Ph.D., and Tamás Forster M.D., Ph.D., D.Sc., for their support in my research.

I would like to thank all my colleagues at the Institute of Surgical Research and Department of Cardiac Surgery for their participation in the studies and for thus supporting me in my work and assisting me in obtaining results of better quality.

Lastly, I am very grateful to and wish to thank my family for their love and care as they encouraged me to achieve my goals.

8. REFERENCES

1. **Adrian TE**, Quigley EM, Rose SG, Johnson TJ, Thompson JS. Effects of jejunoileal autotransplantation on gastrointestinal regulatory peptides. Dig Dis Sci. 1994;39:2457-2466.

2. **Alderton WK**, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J. 2001;357:593-615.

3. **Alemayehu A**, Lock KR, Coatney RW, Chou CC. L-NAME, nitric oxide and jejunal motility, blood flow and oxygen uptake in dogs. Br J Pharmacol. 1994;111:205-212.

4. **Altay T**, Gonzales ER, Park TS, Gidday JM. Cerebrovascular inflammation after brief episodic hypoxia: modulation by neuronal and endothelial nitric oxide synthase. J Appl Physiol. 2004;96:1223-1230.

5. **Andoh A**, Fujiyama Y, Araki Y, Kimura T, Tsujikawa T, Bamba T. Role of complement activation and mast cell degranulation in the pathogenesis of rapid intestinal ischemia/reperfusion injury in rats. Digestion. 2001;63:103-107.

6. **Andoh A**, Kimura T, Fukuda M, Araki Y, Fujiyama Y, Bamba T. Rapid intestinal ischaemia-reperfusion injury is suppressed in genetically mast cell-deficient Ws/Ws rats. Clin Exp Immunol. 1999;116:90-93.

7. **Atochin DN**, Clark J, Demchenko IT, Moskowitz MA, Huang PL. Rapid cerebral ischemic preconditioning in mice deficient in endothelial and neuronal nitric oxide synthases. Stroke. 2003;34:1299-1303.

8. **Ballinger WF 2nd**, Christy MG, Ashby WB. Autotrans-plantation of the small intestine: the effect of denervation. Surgery. 1962;52:151-164.

9. **Balsiger BM**, He CL, Zyromski NJ, Sarr MG. Neuronal adrenergic and muscular cholinergic contractile hypersensitivity in canine jejunum after extrinsic denervation. J Gastrointest Surg. 2003;7:572-582.

10. **Barua A**, Standen NB, Galiñanes M. Dual role of nNOS in ischemic injury and preconditioning. BMC Physiol. 2010;10:15.

11. **Beck PL**, Xavier R, Wong J, Ezedi I, Mashimo H, Mizoguchi A, Mizoguchi E, Bhan AK, Podolsky DK. Paradoxical roles of different nitric oxide synthase isoforms in colonic injury. Am J Physiol Gastrointest Liver Physiol. 2004;286:G137-147.

12. **Becker BF**, Kupatt C, Massoudy P, Zahler S. Reactive oxygen species and nitric oxide in myocardial ischemia and reperfusion. Z Kardiol. 2000;89:88-91.

13. **Beckman JS**, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol. 1996;271:C1424-1437.

14. **Beuk RJ**, Tangelder GJ, Maassen RL, Quaedackers JS, Heineman E, Oude Egbrink MG. Leucocyte and platelet adhesion in different layers of the small bowel during experimental total warm ischaemia and reperfusion. Br J Surg. 2008;95:1294-1304.

15. **Bidri M**, Ktorza S, Vouldoukis I, Le Goff L, Debré P, Guillosson JJ, Arock M. Nitric oxide pathway is induced by Fc epsilon RI and up-regulated by stem cell factor in mouse mast cells. Eur J Immunol. 1997;27:2907-2913.

16. **Bischoff SC**. Physiological and pathophysiological functions of intestinal mast cells. Semin Immunopathol. 2009;31:185-205.

17. **Björk J**, Arfors KE. Oxygen free radicals and leukotriene B4 induced increase in vascular leakage is mediated by polymorphonuclear leukocytes. Agents Actions Suppl. 1982;11:63-72.

18. **Blennerhassett MG**, Tomioka M, Bienenstock J. Formation of contacts between mast cells and sympathetic neurons in vitro. Cell Tissue Res. 1991;265:121-128.

19. **Boros M**, Kaszaki J, Nagy S. Histamine release during intestinal ischemia-reperfusion: role of iron ions and hydrogen peroxide. Circ Shock. 1991;35:174-180.

20. **Boros M**, Takaichi S, Masuda J, Newlands GF, Hatanaka K. Response of mucosal mast cells to intestinal ischemia-reperfusion injury in the rat. Shock. 1995;3:125-131.

21. **Brookes SJ**. Neuronal nitric oxide in the gut. J Gastroenterol Hepatol. 1993;8:590-603.

22. **Buhner S**, Schemann M. Mast cell-nerve axis with a focus on the human gut. Biochim Biophys Acta. 2012;1822:85-92.

23. **Calcina F**, Barocelli E, Bertoni S, Furukawa O, Kaunitz J, Impicciatore M, Sternini C. Effect of N-methyl-d-aspartate receptor blockade on neuronal plasticity and gastrointestinal transit delay induced by ischemia/reperfusion in rats. Neuroscience. 2005;134:39-49.

24. **Camprodon RA**, Bowles MJ, Pockley AG, de Oca J. Anti-inflammatory effects of ischemic preconditioning on rat small bowel allografts. Transplant Proc. 2014;46:2146-2149.

25. **Cao B**, Li N, Wang Y, Li JS. Protective effect of L-arginine preconditioning on ischemia and reperfusion injury associated with rat small bowel transplantation. World J Gastroenterol. 2005;11:2994-2997.

26. **Carmody IC**, Meng L, Shen XD, Anselmo D, Gao F, Ke B, Ma JP, Kupiec-Weglinski JW, McDiarmid SV, Busuttil RW, Shaw G, Farmer DG. P-selectin knockout mice have improved outcomes with both warm ischemia and small bowel transplantation. Transplant Proc. 2004;36:263-264.

27. **Castro M**, Muñoz JM, Arruebo MP, Murillo MD, Arnal C, Bonafonte JI, Plaza MA. Involvement of neuronal nitric oxide synthase (nNOS) in the regulation of migrating motor complex (MMC) in sheep. Vet J. 2012;192:352-358.

28. **Cerutti C**, Ridley AJ. Endothelial cell-cell adhesion and signaling. Exp Cell Res. 2017;358:31-38.

29. **Chiu CJ**, McArdle AH, Brown R, Scott HJ, Gurd FN. Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. Arch Surg. 1970;101:478-483.

30. **Cicalese L**, Caraceni P, Nalesnik MA, Borle AB, Schraut WH. Oxygen free radical content and neutrophil infiltration are important determinants in mucosal injury after rat small bowel transplantation. Transplantation. 1996;62:161-166.

31. **Colgan SP**, Eltzschig HK. Adenosine and hypoxia-inducible factor signaling in intestinal injury and recovery. Annu Rev Physiol. 2012;74:153-175.

32. **Colgan SP**. Neutrophils and inflammatory resolution in the mucosa. Semin Immunol. 2015;27177-183.

33. **Cook-Mills JM**, Marchese ME, Abdala-Valencia H. Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants. Antioxid Redox Signal. 2011;15:1607-1638.

34. **Cooper D**, Russell J, Chitman KD, Williams MC, Wolf RE, Granger DN. Leukocyte dependence of platelet adhesion in postcapillary venules. Am J Physiol Heart Circ Physiol. 2004;286:H1895-1900.

35. **Cordeiro PG**, Lee JJ, Mastorakos D, Hu QY, Pinto JT, Santamaria E. Prevention of ischemia-reperfusion injury in a rat skin flap model: the role of mast cells, cromolyn sodium, and histamine receptor blockade. Plast Reconstr Surg. 2000;105:654-659.

36. **Cuzzocrea S**, Zingarelli B, Caputi AP. Role of constitutive nitric oxide synthase and peroxynitrite production in a rat model of splanchnic artery occlusion shock. Life Sci. 1998;63:789-799.

37. **De Filippo K**, Dudeck A, Hasenberg M, Nye E, van Rooijen N, Hartmann K, Gunzer M, Roers A, Hogg N. Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. Blood. 2013;121:4930-4937.

38. **de Groot H**, Rauen U. Ischemia-reperfusion injury: processes in pathogenetic networks: a review. Transplant Proc. 2007;39:481-484.

39. **de Oca J**, Hotter G, Sola A, González R, Rafecas A, Roselló J, Jaurrieta E. Role of nitric oxide in preconditioning for intestinal transplantation. Transplant Proc. 1999;31:2573.

40. **Dejana E**, Tournier-Lasserve E, Weinstein BM. The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. Dev Cell. 2009;16:209-221.

41. **Dong Z**, Saikumar P, Weinberg JM, Venkatachalam MA. Calcium in cell injury and death. Annu Rev Pathol. 2006;1:405-434.

42. **Ehara T**, Shigematsu H. Mast cells in the kidney. Nephrology (Carlton). 2003;8:130-138.

43. **Emre A**, Bayram O, Salman B, Ercan S, Anadol Z, Akin O. Sodium nitroprusside as a nitric oxide donor in a rat intestinal ischemia-reperfusion model. Clinics (Sao Paulo). 2008;63:91-96.

44. **Enkhbaatar P**, Lange M, Nakano Y, Hamahata A, Jonkam C, Wang J, Jaroch S, Traber L, Herndon D, Traber D. Role of neuronal nitric oxide synthase in ovine sepsis model. Shock. 2009;32:253-257.

45. **Ferencz A**, Szántó Z, Borsiczky B, Kiss K, Kalmár-Nagy K, Szeberényi J, Horváth PO, Róth E. The effects of preconditioning on the oxidative stress in small-bowel autotransplantation. Surgery. 2002;132:877-884.

46. **Ferencz A**, Szántó Z, Kalmár-Nagy K, Horváth OP, Rõth E. Mitigation of oxidative injury by classic and delayed ischemic preconditioning prior to small bowel autotransplantation. Transplant Proc. 2004;36:286-288.

47. **Ferrer-Sueta G**, Radi R. Chemical biology of peroxynitrite: kinetics, diffusion, and radicals. ACS Chem Biol. 2009;4:161-177.

48. **Filpa V**, Carpanese E, Marchet S, Pirrone C, Conti A, Rainero A, Moro E, Chiaravalli AM, Zucchi I, Moriondo A, Negrini D, Crema F, Frigo G, Giaroni C, Porta G. Nitric oxide regulates homeoprotein OTX1 and OTX2 expression in the rat myenteric plexus after intestinal ischemia-reperfusion injury. Am J Physiol Gastrointest Liver Physiol. 2017;312:G374-G389.

49. **Foster MW**, Hess DT, Stamler JS. Protein S-nitrosylation in health and disease: a current perspective. Trends Mol Med. 2009;15:391-404.

50. **Fozard JR**, Pfannkuche HJ, Schuurman HJ. Mast cell degranulation following adenosine A3 receptor activation in rats. Eur J Pharmacol. 1996;298:293-297.

51. **Fukuoka Y**, Hugli TE. Anaphylatoxin binding and degradation by rat peritoneal mast cells. Mechanisms of degranulation and control. J Immunol. 1990;145:1851-1858.

52. **Furness JB**, Callaghan BP, Rivera LR, Cho HJ. The enteric nervous system and gastrointestinal innervation: integrated local and central control. Adv Exp Med Biol. 2014;817:39-71.

53. **Gaboury J**, Woodman RC, Granger DN, Reinhardt P, Kubes P. Nitric oxide prevents leukocyte adherence: role of superoxide. Am J Physiol. 1993;265:H862-867.

54. **Gaboury JP**, Niu XF, Kubes P. Nitric oxide inhibits numerous features of mast cell-induced inflammation. Circulation. 1996;93:318-326.

55. **Galli SJ**, Grimbaldeston M, Tsai M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. Nat Rev Immunol. 2008;8:478-486.

56. **Galli SJ**, Kalesnikoff J, Grimbaldeston MA, Piliponsky AM, Williams CM, Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: recent advances. Annu Rev Immunol. 2005;23:749-786.

57. **Gan X**, Liu D, Ge M, Luo C, Gao W, Hei Z. Treatment of mice with cromolyn sodium after reperfusion, but not prior to ischemia, attenuates small intestinal ischemia-reperfusion injury. Mol Med Rep. 2013;8:928-934.

58. **Gayle J**, Jones SL, Argenzio RA, Blikslager AT. Neutrophils increase paracellular permeability of restituted ischemic-injured porcine ileum. Surgery. 2002;132:461-470.

59. **Gera L**, Varga R, Török L, Kaszaki J, Szabó A, Nagy K, Boros M. Beneficial effects of phosphatidylcholine during hindlimb reperfusion. J Surg Res. 2007;139:45-50.

60. **Giaroni C**, Marchet S, Carpanese E, Prandoni V, Oldrini R, Bartolini B, Moro E, Vigetti D, Crema F, Lecchini S, Frigo G. Role of neuronal and inducible nitric oxide synthases in the guinea pig ileum myenteric plexus during in vitro ischemia and reperfusion. Neurogastroenterol Motil. 2013;25:e114-126.

61. **Gil V**, Gallego D, Grasa L, Martín MT, Jiménez M. Purinergic and nitrergic neuromuscular transmission mediates spontaneous neuronal activity in the rat colon. Am J Physiol Gastrointest Liver Physiol. 2010;299:G158-169.

62. **Ginzberg HH**, Cherapanov V, Dong Q, Cantin A, McCulloch CA, Shannon PT, Downey GP. Neutrophil-mediated epithelial injury during transmigration: role of elastase. Am J Physiol Gastrointest Liver Physiol. 2001;281:G705-717.

63. **Goldstein DR**, Palmer SM. Role of Toll-like receptor-driven innate immunity in thoracic organ transplantation. J Heart Lung Transplant. 2005;24:1721-1729.

64. **Gonzalez AP**, Sepulveda S, Massberg S, Baumeister R, Menger MD. In vivo fluorescence microscopy for the assessment of microvascular reperfusion injury in small bowel transplants in rats. Transplantation. 1994;58:403-408.

65. **Gow AJ**, Farkouh CR, Munson DA, Posencheg MA, Ischiropoulos H. Biological significance of nitric oxide-mediated protein modifications. Am J Physiol Lung Cell Mol Physiol. 2004;287:L262-268.

66. **Granger DN**, Höllwarth ME, Parks DA. Ischemia-reperfusion injury: role of oxygen-derived free radicals. Acta Physiol Scand Suppl. 1986;548:47-63

67. **Granger DN**. Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. Am J Physiol. 1988;255:H1269-1275.

68. **Grisham MB**, Granger DN, Lefer DJ. Modulation of leukocyte-endothelial interactions by reactive metabolites of oxygen and nitrogen: relevance to ischemic heart disease. Free Radic Biol Med. 1998;25:404-433.

69. **Grisham MB**, Hernandez LA, Granger DN. Xanthine oxidase and neutrophil infiltration in intestinal ischemia. Am J Physiol. 1986;251:G567-574.

70. **Grossie VB Jr**, Weisbrodt NW, Moore FA, Moody F. Ischemia/reperfusion-induced disruption of rat small intestine transit is reversed by total enteral nutrition. Nutrition. 2001;17:939-943.

71. **Guan Y**, Worrell RT, Pritts TA, Montrose MH. Intestinal ischemia-reperfusion injury: reversible and irreversible damage imaged in vivo. Am J Physiol Gastrointest Liver Physiol. 2009;297:G187-196.

72. **Haglund U**. Systemic mediators released from the gut in critical illness. Crit Care Med. 1993;21:S15-18.

73. **Hakgüder G**, Akgür FM, Ateş O, Olguner M, Aktuğ T, Ozer E. Short-term intestinal ischemia-reperfusion alters intestinal motility that can be preserved by xanthine oxidase inhibition. Dig Dis Sci. 2002;47:1279-1283.

74. **Harris AG**, Leiderer R, Peer F, Messmer K. Skeletal muscle microvascular and tissue injury after varying durations of ischemia. Am J Physiol. 1996;271:H2388-2398.

75. **Haskó G**, Linden J, Cronstein B, Pacher P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat Rev Drug Discov. 2008 Sep;7(9):759-70. doi: 10.1038/nrd2638.

76. **Hei ZQ**, Gan XL, Luo GJ, Li SR, Cai J. Pretreatment of cromolyn sodium prior to reperfusion attenuates early reperfusion injury after the small intestine ischemia in rats. World J Gastroenterol. 2007;13:5139-5146.

77. **Hirji NS**, Lin TJ, Gilchrist M, Nault G, Nohara O, Grill BJ, Belosevic M, Stenton GR, Schreiber AD, Befus AD. Novel CD8 molecule on macrophages and mast cells: expression, function and signaling. Int Arch Allergy Immunol. 1999;118:180-182.

78. **Hotter G**, Closa D, Prados M, Fernández-Cruz L, Prats N, Gelpí E, Roselló-Catafau J. Intestinal preconditioning is mediated by a transient increase in nitric oxide. Biochem Biophys Res Commun. 1996;222:27-32.

79. **Huet PM**, Nagaoka MR, Desbiens G, Tarrab E, Brault A, Bralet MP, Bilodeau M. Sinusoidal endothelial cell and hepatocyte death following cold ischemia-warm reperfusion of the rat liver. Hepatology. 2004;39:1110-1119.

80. **Huge A**, Kreis ME, Jehle EC, Ehrlein HJ, Starlinger M, Becker HD, Zittel TT. A model to investigate postoperative ileus with strain gauge transducers in awake rats. J Surg Res. 1998;74:112-118.

81. **Hutcheson IR**, Whittle BJ, Boughton-Smith NK. Role of nitric oxide in maintaining vascular integrity in endotoxin-induced acute intestinal damage in the rat. Br J Pharmacol. 1990;101:815-820.

82. **Iba T**, Kidokoro A, Fukunaga M, Takuhiro K, Ouchi M, Ito Y. Comparison of the protective effects of type III phosphodiesterase (PDE3) inhibitor (cilostazol) and acetylsalicylic acid on intestinal microcirculation after ischemia reperfusion injury in mice. Shock. 2006;26:522-526.

83. **Iijima H**, Tulic MK, Duguet A, Shan J, Carbonara P, Hamid Q, Eidelman DH. NOS 1 is required for allergen-induced expression of NOS 2 in mice. Int Arch Allergy Immunol. 2005;138:40-50.

84. **Iikura M**, Takaishi T, Hirai K, Yamada H, Iida M, Koshino T, Morita Y. Exogenous nitric oxide regulates the degranulation of human basophils and rat peritoneal mast cells. Int Arch Allergy Immunol. 1998;115:129-136.

85. **Ikeda T**, Yanaga K, Kishikawa K, Kakizoe S, Shimada M, Sugimachi K. Ischemic injury in liver transplantation: difference in injury sites between warm and cold ischemia in rats. Hepatology. 1992;16:454-461.

86. **Ishii H**, Hashimoto N, Kitada T, Kuroki T, Utsnomiya J. Malabsorption after small-bowel transplantation. Hepatogastroenterology. 1993;40:282-284.

87. **Iuliano L**, Pedersen JZ, Praticò D, Rotilio G, Violi F. Role of hydroxyl radicals in the activation of human platelets. Eur J Biochem. 1994;221:695-704.

88. **Iwanami K**, Takeyoshi I, Ohwada S, Kobayashi J, Kawata K, Matsumoto K, Morishita Y. Intramucosal pH and intestinal mucosal damage in ischemia-reperfusion injury. Transpl Int. 1998;11:401-407.

89. **Jansson L**, Carlsson PO, Bodin B, Andersson A, Källskog O. Neuronal nitric oxide synthase and splanchnic blood flow in anaesthetized rats. Acta Physiol Scand. 2005;183:257-262.

90. **Ji YY**, Wang ZD, Wang SF, Wang BT, Yang ZA, Zhou XR, Lei NN, Yue WN. Ischemic preconditioning ameliorates intestinal injury induced by ischemia-reperfusion in rats. World J Gastroenterol. 2015;21:8081-8088.

91. **Jiang W**, Kirkup AJ, Grundy D. Mast cells drive mesenteric afferent signalling during acute intestinal ischaemia. J Physiol. 2011;589:3867-3882.

92. **Johnson CP**, Sarna SK, Zhu YR, Buchmann E, Bonham L, Telford GL, Roza AM, Adams MB. Effects of intestinal transplantation on postprandial motility and regulation of intestinal transit. Surgery. 2001;129:6-14.

93. **Jungraithmayr W**. The putative role of mast cells in lung transplantation. Am J Transplant. 2015;15:594-600.

94. **Kalia N**, Brown NJ, Wood RF, Pockley AG. Ketotifen abrogates local and systemic consequences of rat intestinal ischemia-reperfusion injury. J Gastroenterol Hepatol. 2005;20:1032-1038.

95. **Kang SW**, Kim OK, Seo B, Lee SH, Quan FS, Shin JH, Lee GJ, Park HK. Simultaneous, real-time measurement of nitric oxide and oxygen dynamics during cardiac ischemia-reperfusion of the rat utilizing sol-gel-derived electrochemical microsensors. Anal Chim Acta. 2013;802:74-81.

96. **Kanwar S**, Kubes P. Ischemia/reperfusion-induced granulocyte influx is a multistep process mediated by mast cells. Microcirculation. 1994d;1:175-182.

97. **Kanwar S**, Kubes P. Mast cells contribute to ischemia-reperfusion-induced granulocyte infiltration and intestinal dysfunction. Am J Physiol. 1994c;267:G316-321.

98. **Kanwar S**, Tepperman BL, Payne D, Sutherland LR, Kubes P. Time course of nitric oxide production and epithelial dysfunction during ischemia/reperfusion of the feline small intestine. Circ Shock. 1994b;42:135-140.

99. **Kanwar S**, Wallace JL, Befus D, Kubes P. Nitric oxide synthesis inhibition increases epithelial permeability via mast cells. Am J Physiol. 1994a;266:G222-229.

100. **Kappus M**, Diamond S, Hurt RT, Martindale 4. Intestinal Failure: New Definition and Clinical Implications. Curr Gastroenterol Rep. 2016;18:48.

101. **Karhausen J**, Furuta GT, Tomaszewski JE, Johnson RS, Colgan SP, Haase VH. Epithelial hypoxia-inducible factor-1 is protective in murine experimental colitis. J Clin Invest. 2004 Oct;114(8):1098-106.

102. **Katsanos GS**, Anogeianaki A, Orso C, Tetè S, Salini V, Antinolfi PL, Sabatino G. Mast cells and chemokines. J Biol Regul Homeost Agents. 2008;22:145-151.

103. **Katsube T**, Tsuji H, Onoda M. Nitric oxide attenuates hydrogen peroxide-induced barrier disruption and protein tyrosine phosphorylation in monolayers of intestinal epithelial cell. Biochim Biophys Acta. 2007;1773:794-803.

104. **Kawata K**, Takeyoshi I, Iwanami K, Sunose Y, Aiba M, Ohwada S, Matsumoto K, Morishita Y. A spontaneous nitric oxide donor ameliorates small bowel ischemia-reperfusion injury in dogs. Dig Dis Sci. 2001;46:1748-1756.

105. **Khan BV**, Harrison DG, Olbrych MT, Alexander RW, Medford RM. Nitric oxide regulates vascular cell adhesion molecule 1 gene expression and redox-sensitive transcriptional events in human vascular endothelial cells. Proc Natl Acad Sci U S A. 1996;93:9114-9119.

106. **Knowles RG**, Moncada S. Nitric oxide synthases in mammals. Biochem J. 1994;298:249-258.

107. **Kono T**, Saito M, Kinoshita Y, Satoh I, Shinbori C, Satoh K. Real-time monitoring of nitric oxide and blood flow during ischemia-reperfusion in the rat testis. Mol Cell Biochem. 2006;286:139-145.

108. **Kubes P**, Gaboury JP. Rapid mast cell activation causes leukocyte-dependent and -independent permeability alterations. Am J Physiol. 1996;271:H2438-2446.

109. **Kubes P**, Kanwar S, Niu XF, Gaboury JP. Nitric oxide synthesis inhibition induces leukocyte adhesion via superoxide and mast cells. FASEB J. 1993a;7:1293-1299.

110. **Kubes P**, McCafferty DM. Nitric oxide and intestinal inflammation. Am J Med. 2000;109:150-158.

111. **Kubes P**, Suzuki M, Granger DN. Nitric oxide: an endogenous modulator of leukocyte adhesion. Proc Natl Acad Sci U S A. 1991;88:4651-4655.

112. **Kubes P**. Ischemia-reperfusion in feline small intestine: a role for nitric oxide. Am J Physiol. 1993b;264:G143-149.

113. **Kuebler WM**, Abels C, Schuerer L, Goetz AE. Measurement of neutrophil content in brain and lung tissue by a modified myeloperoxidase assay. Int J Microcirc Clin Exp. 1996;16:89-97.

114. **Kuehn HS**, Gilfillan AM. G protein-coupled receptors and the modification of FcepsilonRI-mediated mast cell activation. Immunol Lett. 2007;113:59-69.

115. **Kunder CA**, St John AL, Abraham SN. Mast cell modulation of the vascular and lymphatic endothelium. Blood. 2011;118:5383-5393.

116. **Kurose I**, Argenbright LW, Wolf R, Lianxi L, Granger DN. Ischemia/reperfusion-induced microvascular dysfunction: role of oxidants and lipid mediators. Am J Physiol. 1997;272:H2976-2982.

117. **Lai CH,** Lee CH, Hung CY, Lo HC. Oral Citrulline Mitigates Inflammation and Jejunal Damage via the Inactivation of Neuronal Nitric Oxide Synthase and Nuclear Factor-κB in Intestinal Ischemia and Reperfusion. JPEN J Parenter Enteral Nutr. 2017 ;41:422-435.

118. **Land WG**. Immunosuppressive strategies in organ transplantation in the light of innate immunity. Exp Clin Transplant. 2006;4:406-415.

119. **Land WG**.Innate immunity-mediated allograft rejection and strategies to prevent it. Transplant Proc. 2007;39:667-672.

120. **Lautenschläger I**, Pless-Petig G, Middel P, de Groot H, Rauen U, Stojanovic T. Cold Storage Injury to Rat Small-bowel Transplants-Beneficial Effect of a Modified HTK Solution. Transplantation. 2018;102:1666-1673.

121. **Lee S**, Chung J, Ha IS, Yi K, Lee JE, Kang HG, Choi I, Oh KH, Kim JY, Surh CD, Ahn C. Hydrogen peroxide increases human leukocyte adhesion to porcine aortic

endothelial cells via NFkappaB-dependent up-regulation of VCAM-1. Int Immunol. 2007;19:1349-1359.

122. **Lefer DJ**, Granger DN. Oxidative stress and cardiac disease. Am J Med. 2000;109:315-323.

123. **Legendre C**, Canaud G, Martinez F. Factors influencing long-term outcome after kidney transplantation. Transpl Int. 2014;27:19-27.

124. **Leo R**, Praticò D, Iuliano L, Pulcinelli FM, Ghiselli A, Pignatelli P, Colavita AR, FitzGerald GA, Violi F. Platelet activation by superoxide anion and hydroxyl radicals intrinsically generated by platelets that had undergone anoxia and then reoxygenated. Circulation. 1997;95:885-891.

125. **Ley K**, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol. 2007;7:678-689.

126. **Lhuillier F**, Parmantier P, Goudable J, Crova P, Delafosse B, Annat G, Cespuglio R, Viale JP. Hepatic ischemia is associated with an increase in liver parenchyma nitric oxide that is in part enzyme-independent. Anesthesiology. 2003;98:373-378.

127. **Li H**, Cui H, Kundu TK, Alzawahra W, Zweier JL. Nitric oxide production from nitrite occurs primarily in tissues not in the blood: critical role of xanthine oxidase and aldehyde oxidase. J Biol Chem. 2008;283:17855-17863.

128. **Li H**, Samouilov A, Liu X, Zweier JL. Characterization of the magnitude and kinetics of xanthine oxidase-catalyzed nitrite reduction. Evaluation of its role in nitric oxide generation in anoxic tissues. J Biol Chem. 2001;276:24482-24489.

129. **Lillehei RC**, Goott B, Miller FA. The physiological response of the small bowel of the dog to ischemia including prolonged in vitro preservation of the bowel with successful replacement and survival. Ann Surg. 1959;150:543-560.

130. **Lillehei RC**, Idezuki Y, Feemster JA, Dietzman RH, Kelly WD, Merkel FK, Goetz FC, Lyons GW, Manax WG. Transplantation of stomach, intestine, and pancreas: experimental and clinical observations. Surgery. 1967;62:721-741.

131. **Loo L**, Vrakas G, Reddy S, Allan P. Intestinal transplantation: a review. Curr Opin Gastroenterol. 2017;33:203-211.

132. **Lu XM**, Zhang GX, Yu YQ, Kimura S, Nishiyama A, Matsuyoshi H, Shimizu J, Takaki M. The opposite roles of nNOS in cardiac ischemia-reperfusion-induced injury and in ischemia preconditioning-induced cardioprotection in mice. J Physiol Sci. 2009;59:253-262.

133. **Mallick IH**, Yang W, Winslet MC, Seifalian AM. Ischaemic preconditioning improves microvascular perfusion and oxygenation following reperfusion injury of the intestine. Br J Surg. 2005a;92:1169-1176.

134. **Mallick IH**, Yang W, Winslet MC, Seifalian AM. Ischemia-reperfusion injury of the intestine and protective strategies against injury. Dig Dis Sci. 2004;49:1359-1377.

135. **Mallick IH**, Yang W, Winslet MC, Seifalian AM. Protective effects of ischemic preconditioning on the intestinal mucosal microcirculation following ischemia-reperfusion of the intestine. Microcirculation. 2005b;12:615-625.

136. **Maretta M**, Tóth Š, Bujdoš M, Tóth Š Jr, Jonecová Z, Veselá J. Alterations of epithelial layer after ischemic preconditioning of small intestine in rats. J Mol Histol. 2012;43:171-178.

137. **Marui N**, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, Medford RM. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. J Clin Invest. 1993;92:1866-1874.

138. **Massberg S**, Gonzalez AP, Leiderer R, Menger MD, Messmer K. In vivo assessment of the influence of cold preservation time on microvascular reperfusion injury after experimental small bowel transplantation. Br J Surg. 1998;85:127-133.

139. **Matsui T**, Suzuki Y, Yamashita K, Yoshimaru T, Suzuki-Karasaki M, Hayakawa S, Yamaki M, Shimizu K. Diphenyleneiodonium prevents reactive oxygen species generation, tyrosine phosphorylation, and histamine release in RBL-2H3 mast cells. Biochem Biophys Res Commun. 2000;276:742-748.

140. **Matsuura T**, Masumoto K, Ieiri S, Nakatsuji T, Akiyoshi J, Nishimoto Y, Takahashi Y, Hayashida M, Taguchi T. Morphological and physiological changes of interstitial cells of Cajal after small bowel transplantation in rats. Transpl Int. 2007;20:616-624.

141. **Maulik N**, Goswami S, Galang N, Das DK. Differential regulation of Bcl-2, AP-1 and NF-kappaB on cardiomyocyte apoptosis during myocardial ischemic stress adaptation. FEBS Lett. 1999;443:331-336.

142. **McCallion K**, Wattanasirichaigoon S, Gardiner KR, Fink MP. Ischemic preconditioning ameliorates ischemia- and reperfusion-induced intestinal epithelial hyperpermeability in rats. Shock. 2000;14:429-434.

143. **McCartney SL**, Patel C, Del Rio JM. Long-term outcomes and management of the heart transplant recipient. Best Pract Res Clin Anaesthesiol. 2017;31:237-248.

144. **McCord JM**. Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med. 1985;312:159-163.

145. **Menger MD**, Vollmar B. Role of microcirculation in transplantation. Microcirculation. 2000;7:291-306.

146. **Moore PK**, Bland-Ward PA. 7-nitroindazole: an inhibitor of nitric oxide synthase. Methods Enzymol. 1996;268:393-398.

147. **Moore-Olufemi SD**, Kozar RA, Moore FA, Sato N, Hassoun HT, Cox CS Jr, Kone BC. Ischemic preconditioning protects against gut dysfunction and mucosal injury after ischemia/reperfusion injury. Shock. 2005;23:258-263.

148. **Morita H**, Mochiki E, Ogawa A, Yanai M, Toyomasu Y, Tabe Y, Ohno T, Tsutsumi S, Asao T, Kuwano H. Effects of denervation at ileocecal junction and ileocecal resection in dogs. Neurogastroenterol Motil. 2012;24:86-93,e14.

149. **Mu K**, Yu S, Kitts DD. The Role of Nitric Oxide in Regulating Intestinal Redox Status and Intestinal Epithelial Cell Functionality. Int J Mol Sci. 2019;20. pii: E1755.

150. **Muller WA**. Mechanisms of leukocyte transendothelial migration. Annu Rev Pathol. 2011;6:323-344.

151. **Murray DB**, Gardner JD, Brower GL, Janicki JS. Endothelin-1 mediates cardiac mast cell degranulation, matrix metalloproteinase activation, and myocardial remodeling in rats. Am J Physiol Heart Circ Physiol. 2004;287:H2295-2299.

152. **Murry CE**, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation. 1986;74:1124-1136.

153. **Nair J**, Lakshminrusimha S. Role of NO and other vascular mediators in the etiopathogenesis of necrotizing enterocolitis. Front Biosci (Schol Ed). 2019;11:9-28.

154. **Nakamura T**, Lipton SA. S-Nitrosylation and uncompetitive/fast off-rate (UFO) drug therapy in neurodegenerative disorders of protein misfolding. Cell Death Differ. 2007;14:1305-1314.

155. **Nash S**, Stafford J, Madara JL. Effects of polymorphonuclear leukocyte transmigration on the barrier function of cultured intestinal epithelial monolayers. J Clin Invest. 1987;80:1104-1113.

156. **Nelson DK**, Furlow BL, Go VL, Sarr MG. Enteroendocrine peptides in a canine model of orthotopic jejunoileal autotransplantation. Regul Pept. 1993;45:421-434.

157. **Neuberger J**. An update on liver transplantation: A critical review. J Autoimmun. 2016;66:51-59.

158. **Nieuwenhuijs VB**, Verheem A, van Duijvenbode-Beumer H, Visser MR, Verhoef J, Gooszen HG, Akkermans LM. The role of interdigestive small bowel motility in the regulation of gut microflora, bacterial overgrowth, and bacterial translocation in rats. Ann Surg. 1998;228:188-193.

159. **Niu XF**, Ibbotson G, Kubes P. A balance between nitric oxide and oxidants regulates mast cell-dependent neutrophil-endothelial cell interactions. Circ Res. 1996;79:992-999.

160. **Nusrat A**, Parkos CA, Liang TW, Carnes DK, Madara JL. Neutrophil migration across model intestinal epithelia: monolayer disruption and subsequent events in epithelial repair. Gastroenterology. 1997;113:1489-1500.

161. **Ohta K**, Graf R, Rosner G, Kumura E, Heiss WD. Early nitric oxide increase in depolarized tissue of cat focal cerebral ischaemia. Neuroreport. 1996;8:143-148.

162. **Oishi AJ**, Sarr MG. Intestinal transplantation: effects on ileal enteric absorptive physiology. Surgery. 1995;117:545-553.

163. **O'Keefe SJ**, Buchman AL, Fishbein TM, Jeejeebhoy KN, Jeppesen PB, Shaffer J. Short bowel syndrome and intestinal failure: consensus definitions and overview. Clin Gastroenterol Hepatol. 2006;4:6-10.

164. **Palásthy Z**, Kaszaki J, Lázár G, Nagy S, Boros M. Intestinal nitric oxide synthase activity changes during experimental colon obstruction. Scand J Gastroenterol. 2006;41:910-918.

165. **Palombit K**, Mendes CE, Tavares-de-Lima W, Silveira MP, Castelucci P. Effects of ischemia and reperfusion on subpopulations of rat enteric neurons expressing the P2X7 receptor. Dig Dis Sci. 2013;58:3429-3439.

166. **Panés J**, Perry M, Granger DN. Leukocyte-endothelial cell adhesion: avenues for therapeutic intervention. Br J Pharmacol. 1999;126:537-550.

167. **Parikh V**, Singh M. Cardiac mast cell stabilization and cardioprotective effect of ischemic preconditioning in isolated rat heart. J Cardiovasc Pharmacol. 1998;31:779-785.

168. **Parks DA**, Bulkley GB, Granger DN, Hamilton SR, McCord JM. Ischemic injury in the cat small intestine: role of superoxide radicals. Gastroenterology. 1982;82:9-15.

169. **Parks DA**, Granger DN. Ischemia-induced vascular changes: role of xanthine oxidase and hydroxyl radicals. Am J Physiol. 1983;245:G285-289.

170. **Parks DA**, Williams TK, Beckman JS. Conversion of xanthine dehydrogenase to oxidase in ischemic rat intestine: a reevaluation. Am J Physiol. 1988;254:G768-774.

171. **Park-Windhol C**, D'Amore PA. Disorders of Vascular Permeability. Annu Rev Pathol. 2016;11:251-281.

172. **Payne D**, Kubes P. Nitric oxide donors reduce the rise in reperfusion-induced intestinal mucosal permeability. Am J Physiol. 1993;265:G189-195.

173. **Pober JS**, Sessa WC. Evolving functions of endothelial cells in inflammation. Nat Rev Immunol. 2007;7:803-815.

174. **Pohlman TH**, Stanness KA, Beatty PG, Ochs HD, Harlan JM. An endothelial cell surface factor(s) induced in vitro by lipopolysaccharide, interleukin 1, and tumor necrosis factor-alpha increases neutrophil adherence by a CDw18-dependent mechanism. J Immunol. 1986;136:4548-4553.

175. **Pontell L**, Sharma P, Rivera LR, Thacker M, Tan YH, Brock JA, Furness JB. Damaging effects of ischemia/reperfusion on intestinal muscle. Cell Tissue Res. 2011;343:411-419.

176. **Preissler G**, Massberg S, Enders G, Waldner H, Messmer K. Effect of hypothermic preservation on ICAM-1 mRNA induction, leukocyte-endothelium interaction and microvascular perfusion after pancreas transplantation in the rat. Langenbecks Arch Chir Suppl Kongressbd. 1998;115:161-164.

177. **Qu XW**, Rozenfeld RA, Huang W, Sun X, Tan XD, Hsueh W. Roles of nitric oxide synthases in platelet-activating factor-induced intestinal necrosis in rats. Crit Care Med. 1999a;27:356-364.

178. **Qu XW**, Thaete LG, Rozenfeld RA, Zhu Y, De Plaen IG, Caplan MS, Hsueh W. Tetrahydrobiopterin prevents platelet-activating factor-induced intestinal hypoperfusion and necrosis: Role of neuronal nitric oxide synthase. Crit Care Med. 2005;33:1050-1056.

179. **Qu XW**, Wang H, De Plaen IG, Rozenfeld RA, Hsueh W. Neuronal nitric oxide synthase (NOS) regulates the expression of inducible NOS in rat small intestine via modulation of nuclear factor kappa B. FASEB J. 2001;15:439-446.

180. **Qu XW**, Wang H, Rozenfeld RA, Huang W, Hsueh W. Type I nitric oxide synthase (NOS) is the predominant NOS in rat small intestine. Regulation by platelet-activating factor. Biochim Biophys Acta. 1999b;1451:211-217.

181. **Quigley EM**, Thompson JS. The effects of surgery on gastrointestinal motor activity. Braz J Med Biol Res. 1998l;31:889-900.

182. **Rauen U**, de Groot H. New insights into the cellular and molecular mechanisms of cold storage injury. J Investig Med. 2004;52:299-309.

183. **Rivera LR**, Pontell L, Cho HJ, Castelucci P, Thacker M, Poole DP, Frugier T, Furness JB. Knock out of neuronal nitric oxide synthase exacerbates intestinal ischemia/reperfusion injury in mice. Cell Tissue Res. 2012;349:565-576.

184. **Rivera LR**, Poole DP, Thacker M, Furness JB. The involvement of nitric oxide synthase neurons in enteric neuropathies. Neurogastroenterol Motil. 2011b;23:980-988.

185. **Rivera LR**, Thacker M, Castelucci P, Bron R, Furness JB. The reactions of specific neuron types to intestinal ischemia in the guinea pig enteric nervous system. Acta Neuropathol. 2009;118:261-270.

186. **Rivera LR**, Thacker M, Pontell L, Cho HJ, Furness JB. Deleterious effects of intestinal ischemia/reperfusion injury in the mouse enteric nervous system are associated with protein nitrosylation. Cell Tissue Res. 2011a;344:111-123.

187. **Rybicka JM**, Balce DR, Khan MF, Krohn RM, Yates RM. NADPH oxidase activity controls phagosomal proteolysis in macrophages through modulation of the lumenal redox environment of phagosomes. Proc Natl Acad Sci U S A. 2010;107:10496-10501.

188. **Saeki I**, Matsuura T, Hayashida M, Taguchi T. Ischemic preconditioning and remote ischemic preconditioning have protective effect against cold ischemia-reperfusion injury of rat small intestine. Pediatr Surg Int. 2011;27:857-862.

189. **Saito M**, Miyagawa I. Direct detection of nitric oxide in rat urinary bladder during ischemia-reperfusion. J Urol. 1999;162:1490-1495.

190. **Saito M**, Miyagawa I. Real-time monitoring of nitric oxide in ischemia-reperfusion rat kidney. Urol Res. 2000;28:141-146.

191. **Salzman AL**. Nitric oxide in the gut. New Horiz. 1995;3:33-45.

192. **Sanz MJ**, Hickey MJ, Johnston B, McCafferty DM, Raharjo E, Huang PL, Kubes P. Neuronal nitric oxide synthase (NOS) regulates leukocyte-endothelial cell interactions in endothelial NOS deficient mice. Br J Pharmacol. 2001;134:305-312.

193. **Savidge TC**. S-nitrosothiol signals in the enteric nervous system: lessons learnt from big brother. Front Neurosci. 2011;5:31.

194. **Schaefer N**, Tahara K, Schmidt J, Wehner S, Kalff JC, Abu-Elmagd K, Hirner A, Türler A. Resident macrophages are involved in intestinal transplantation-associated inflammation and motoric dysfunction of the graft muscularis. Am J Transplant. 2007;7:1062-1070.

195. **Schafer FQ**, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med. 2001;30:1191-1212.

196. **Schemann M**, Camilleri M. Functions and imaging of mast cell and neural axis of the gut. Gastroenterology. 2013;144:698-704.

197. **Schimmel L**, Heemskerk N, van Buul JD. Leukocyte transendothelial migration: A local affair. Small GTPases. 2017;8:1-15.

198. **Schoenberg MH**, Beger HG. Reperfusion injury after intestinal ischemia. Crit Care Med. 1993;21:1376-1386.

199. **Schramm R**, Menger MD, Kirsch S, Langer F, Harder Y, Hamacher J, Schäfers HJ. The subepicardial microcirculation in heterotopically transplanted mouse hearts: an intravital multifluorescence microscopy study. J Thorac Cardiovasc Surg. 2007;134:210-217.

200. **Sheridan AM**, Bonventre JV. Cell biology and molecular mechanisms of injury in ischemic acute renal failure. Curr Opin Nephrol Hypertens. 2000;9:427-434.

201. **Sileri P**, Sica G, Gentileschi P, Venza M, Manzelli A, Palmieri G, Spagnoli LG, Testa G, Benedetti E, Gaspari AL. Ischemic preconditioning protects intestine from prolonged ischemia. Transplant Proc. 2004;36:283-285.

202. **Sola A**, De Oca J, González R, Prats N, Roselló-Catafau J, Gelpí E, Jaurrieta E, Hotter G. Protective effect of ischemic preconditioning on cold preservation and reperfusion injury associated with rat intestinal transplantation. Ann Surg. 2001;234:98-106.

203. **Sola A**, Hotter G, Prats N, Xaus C, Gelpi E, Roselló-Catafau J. Modification of oxidative stress in response to intestinal preconditioning. Transplantation. 2000;69:767-772.

204. **Stallion A**, Kou TD, Miller KA, Dahms BB, Dudgeon DL, Levine AD. IL-10 is not protective in intestinal ischemia reperfusion injury. J Surg Res. 2002;105:145-152.

205. **Stamler JS**, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, Singel DJ, Loscalzo J. S-nitrosylation of proteins with nitric oxide: synthesis and characterization of biologically active compounds. Proc Natl Acad Sci U S A. 1992;89:444-448.

206. **Starzl TE**, Kaupp HA Jr. Mass homotransplantation of abdominal organs in dogs. Surg Forum. 1960;11:28-30.

207. **Stead RH**, Dixon MF, Bramwell NH, Riddell RH, Bienenstock J. Mast cells are closely apposed to nerves in the human gastrointestinal mucosa. Gastroenterology. 1989;97:575-585.

208. **Strbian D**, Karjalainen-Lindsberg ML, Tatlisumak T, Lindsberg PJ. Cerebral mast cells regulate early ischemic brain swelling and neutrophil accumulation. J Cereb Blood Flow Metab. 2006;26:605-612.

209. **Su M**, Chi EY, Bishop MJ, Henderson WR Jr. Lung mast cells increase in number and degranulate during pulmonary artery occlusion/reperfusion injury in dogs. Am Rev Respir Dis. 1993;147:448-456.

210. **Sugama Y**, Tiruppathi C, offakidevi K, Andersen TT, Fenton JW 2nd, Malik AB. Thrombin-induced expression of endothelial P-selectin and intercellular adhesion molecule-1: a mechanism for stabilizing neutrophil adhesion. J Cell Biol. 1992;119:935-944.

211. **Suzuki M**, Grisham MB, Granger DN. Leukocyte-endothelial cell adhesive interactions: role of xanthine oxidase-derived oxidants. J Leukoc Biol. 1991;50:488-494.

212. **Suzuki M**, Inauen W, Kvietys PR, Grisham MB, Meininger C, Schelling ME, Granger HJ, Granger DN. Superoxide mediates reperfusion-induced leukocyte-endothelial cell interactions. Am J Physiol. 1989;257:H1740-1745.

213. **Suzuki Y**, Yoshimaru T, Inoue T, Niide O, Ra C. Role of oxidants in mast cell activation. Chem Immunol Allergy. 2005;87:32-42.

214. **Szabó A**, Boros M, Kaszaki J, Nagy S. The role of mast cells in mucosal permeability changes during ischemia-reperfusion injury of the small intestine. Shock. 1997;8:284-291.

215. **Szabó A**, Vollmar B, Boros M, Menger MD. Gender differences in ischemia-reperfusion-induced microcirculatory and epithelial dysfunctions in the small intestine. Life Sci. 2006;78:3058-3065.

216. **Szalay L**, Kaszaki J, Nagy S, Boros M. Endothelin-1 induces mucosal mast cell degranulation in the rat small intestine. Life Sci. 2000;67:1947-1958.

217. **Taha MO**, Miranda-Ferreira R, Chang AC, Rodrigues AM, Fonseca IS, Toral LB, Cardoso MR, Simões MJ, Oliveira-Junior IS, Monteiro HP, Fagundes DJ, Taha NS, Caricati-Neto A. Effect of ischemic preconditioning on injuries caused by ischemia and reperfusion in rat intestine. Transplant Proc. 2012;44:2304-2308.

218. **Takahashi SS**, Omori Y, Miyazaki H, Yoshino F, Shoji H, Lee MC, Todoki K, Kamibayashi M, Murakami E. Real-time monitoring of nitric oxide in ischemic myocardium using an NO-selective electrode calibrated by electron spin resonance. Life Sci. 2003;74:75-85.

219. **Takayama I**, Seto E, Zai H, Ohno S, Tezuka H, Daigo Y, Fujino MA. Changes of in vivo gastrointestinal motor pattern in pacemaker-deficient (WsRC-Ws/Ws) rats. Dig Dis Sci. 2000;45:1901-1906.

220. **Tamion F**, Richard V, Lacoume Y, Thuillez C. Intestinal preconditioning prevents systemic inflammatory response in hemorrhagic shock. Role of HO-1. Am J Physiol Gastrointest Liver Physiol. 2002;283:G408-414.

221. **Tegeder I**. Nitric oxide mediated redox regulation of protein homeostasis. Cell Signal. 2019;53:348-356.

222. **Tikoo S**, Barki N, Jain R, Zulkhernain NS, Buhner S, Schemann M, Weninger W- Imaging of mast cells. Immunol Rev. 2018;282:58-72.

223. **Tsiotos GG**, Kendrick ML, Libsch K, Bierens K, Lankisch P, Duenes JA, Sarr MG. Ileal absorptive adaptation to jejunal resection and extrinsic denervation: implications for living-related small bowel transplantation. J Gastrointest Surg. 2001;5:517-524.

224. **Türler A**, Kalff JC, Heeckt P, Abu-Elmagd KM, Schraut WH, Bond GJ, Moore BA, Brünagel G, Bauer AJ. Molecular and functional observations on the donor intestinal muscularis during human small bowel transplantation. Gastroenterology. 2002;122:1886-1897.

225. **Udassin R**, Eimerl D, Schiffman J, Haskel Y. Postischemic intestinal motility in rat is inversely correlated to length of ischemia. An in vivo animal model. Dig Dis Sci. 1995;40:1035-1038.

226. **Unal S**, Demirkan F, Arslan E, Cin I, Cinel L, Eskandari G, Cinel I. Comparison of ischemic and chemical preconditioning in jejunal flaps in the rat. Plast Reconstr Surg. 2003;112:1024-1031.

227. **van Overveld FJ**, Bult H, Vermeire PA, Herman AG. Nitroprusside, a nitrogen oxide generating drug, inhibits release of histamine and tryptase from human skin mast cells. Agents Actions 1993;38:237–238.

228. **Vollmar B**, Menger MD. Intestinal ischemia/reperfusion: microcirculatory pathology and functional consequences. Langenbecks Arch Surg. 2011;396:13-29.

229. **Wada K**, Kamisaki Y, Ohkura T, Kanda G, Nakamoto K, Kishimoto Y, Ashida K, Itoh T. Direct measurement of nitric oxide release in gastric mucosa during ischemia-reperfusion in rats. Am J Physiol. 1998;274:G465-471.

230. **Wan MX**, Wang Y, Liu Q, Schramm R, Thorlacius H. CC chemokines induce P-selectin-dependent neutrophil rolling and recruitment in vivo: intermediary role of mast cells. Br J Pharmacol. 2003;138:698-706.

231. **Wang M**, Qi DS, Zhou C, Han D, Li PP, Zhang F, Zhou XY, Han M, Di JH, Ye JS, Yu HM, Song YJ, Zhang GY. Ischemic preconditioning protects the brain against injury via inhibiting CaMKII-nNOS signaling pathway. Brain Res. 2016;1634:140-149.

232. **Wang S**, Fan L, Gao K, Li G. The protective effect of ischemic preconditioning associated with altered gene expression profiles in intestinal grafts after reperfusion. J Surg Res. 2009;153:340-346.

233. **Wang SF**, Li GW. Early protective effect of ischemic preconditioning on small intestinal graft in rats. World J Gastroenterol. 2003;9:1866-1870.

234. **Wankowicz Z**, Megyeri P, Issekutz A. Synergy between tumour necrosis factor alpha and interleukin-1 in the induction of polymorphonuclear leukocyte migration during inflammation. J Leukoc Biol. 1988;43:349-356.

235. **Watanabe T**, Owada S, Kobayashi H, Ishiuchi A, Nakano H, Asakuta T, Shimamura T, Asano T, Koizumi S, Jinnouchi Y, Katayama M, Kamibayasi M, Murakami E, Otsubo T. Real-time monitoring of nitric oxide (NO) and pO2 levels under ischemic conditions associated with small bowel ischemia/reperfusion injury using selective electrodes for NO and oxygen molecules. Transplant Proc. 2007;39:3007-3009.

236. **Wei Z**, Fan L, Xiangming C. Essential role for nuclear factor kappaB in ischemic preconditioning for cold ischemia-reperfusion injury of intestinal transplantation. Transplant Proc. 2009;41:4120-4122.

237. **Wingard CJ**, Walters DM, Cathey BL, Hilderbrand SC, Katwa P, Lin S, Ke PC, Podila R, Rao A, Lust RM, Brown JM. Mast cells contribute to altered vascular reactivity and ischemia-reperfusion injury following cerium oxide nanoparticle instillation. Nanotoxicology. 2011;5:531-545.

238. **Witthöft T**, Eckmann L, Kim JM, Kagnoff MF. Enteroinvasive bacteria directly activate expression of iNOS and NO production in human colon epithelial cells. Am J Physiol. 1998;275:G564-571.

239. **Wolfárd A**, Szalay L, Kaszaki J, Sahin-Tóth G, Vangel R, Balogh A, Boros M. Dynamic in vivo observation of villus microcirculation during small bowel autotransplantation: effects of endothelin-A receptor inhibition. Transplantation. 2002;73:1511-1513.

240. **Wolfárd A**, Vangel R, Szalay L, Kaszaki J, Haulik L, Balogh A, Nagy S, Boros M. Endothelin-A receptor antagonism improves small bowel graft perfusion and structure after ischemia and reperfusion. Transplantation. 1999;68:1231-1238.

241. **Woodfin A**, Voisin MB, Beyrau M, Colom B, Caille D, Diapouli FM, Nash GB, Chavakis T, Albelda SM, Rainger GE, Meda P, Imhof BA, Nourshargh S. The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. Nat Immunol. 2011;12:761-769.

242. **Xing D**, Zhang R, Li S, Huang P, Luo C, Hei Z, Xia Z, Gan X. Pivotal role of mast cell carboxypeptidase A in mediating protection against small intestinal ischemia-reperfusion injury in rats after ischemic preconditioning. J Surg Res. 2014;192:177-186.

243. **Yang B**, Chen Y, Long YH, Fan X, Liu KX, Wang XB, Zhou J. Intestinal and Limb Ischemic Preconditioning Provides a Combined Protective Effect in the Late Phase, But not in the Early Phase, Against Intestinal Injury Induced by Intestinal Ischemia-Reperfusion in Rats. Shock. 2018;49:596-603.

244. **Yang M**, Ma Y, Ding J, Rao L, Li J. Preconditioning donor livers with cromolyn or compound 48/80 prolongs recipient survival in a rat orthotopic liver transplantation model. Transplant Proc. 2014;46:1554-1559.

245. **Zhang J**, Alcaide P, Liu L, Sun J, He A, Luscinskas FW, Shi GP. Regulation of endothelial cell adhesion molecule expression by mast cells, macrophages, and neutrophils. PLoS One. 2011;6:e14525.

246. **Zhang XQ**, Sun JL, Chen YJ, Ma R, Fan XH, Sun JZ. Amelioration of ischemia-reperfusion injury of transplanted small intestine by ulinastatin: effects on accumulation and adhesion of neutrophil. Transplant Proc. 2005;37:4464-4466.

247. **Zweier JL**, Wang P, Samouilov A, Kuppusamy P. Enzyme-independent formation of nitric oxide in biological tissues. Nat Med. 1995;1:804-809.

248. **Zyromski NJ**, Duenes JA, Kendrick ML, Libsch KD, Seiler R, Tanaka T, Sarr MG. Differential adrenergic response to extrinsic denervation in canine longitudinal jejunal and ileal smooth muscle. J Gastrointest Surg. 2002;6:418-425.

249. **Zyromski NJ**, Duenes JA, Sarr MG. Inhibition by nitric oxide and nonadrenergic, noncholinergic (NANC) nerves is preserved in a canine model of extrinsic denervation: implications for small bowel transplantation. Surgery. 2005;138:905-912.

9. ANNEX

I. Wolfárd A, Kaszaki J, Varga S, Lázár G, Boros M. Early microcirculatory changes after ischemic preconditioning and small bowel autotransplantation. Eur Surg Res. 2007;39:284-290.

European Surgical Research

Early Microcirculatory Changes after Ischemic Preconditioning and Small Bowel Autotransplantation

A. Wolfárd[a, c]   J. Kaszaki[c]   S. Varga[b]   G. Lázár[a]   M. Boros[c]

Departments of [a]Surgery and [b]Cardiac Surgery, [c]Institute of Surgical Research, University of Szeged,
Szent-Györgyi Albert Medical Center, Szeged, Hungary

Abstract

Background/Aims: Ischemia-reperfusion injury contributes to the high complication rate of small bowel transplantation (SBTX). Ischemic preconditioning (IPC) protects against reperfusion injury in several organs, but the IPC-induced microcirculatory reaction in the intestine is unknown. **Methods:** We examined the effects of IPC on the macrohemodynamics and graft microcirculation in a canine model of SBTX during a 4-hour reperfusion period. In group 1 SBTX was performed, in group 2 IPC was induced before graft harvesting (ischemia 3 times for 5 min, followed by 10 min of reperfusion). Cardiac index and mesenteric blood flow were measured, and the mucosal microcirculation, villus epithelial thickness and functional capillary density were monitored by orthogonal polarization spectral imaging. Leukocyte-endothelial cell interactions were monitored in the postcapillary venules, with intravital fluorescence microscopy. **Results:** Reperfusion decreased cardiac index and mesenteric blood flow during reperfusion; IPC significantly improved these changes. Reperfusion was accompanied by decreased functional capillary density and epithelial thickness of the villi and increased leukocyte-endothelial cell interactions. IPC increased functional capillary density, prevented epithelial narrowing and reduced leukocyte rolling and adherence. **Conclusion:** IPC improves the macrohemodynamics and the intestinal microcirculation and reduces leukocyte-mediated tissue injury during reperfusion. IPC can be an effective tool to limit reperfusion injury during SBTX.

Copyright © 2007 S. Karger AG, Basel

Introduction

Small bowel transplantation (SBTX) is the optimal treatment modality and the best alternative to total parenteral nutrition for patients with short bowel syndrome [1–3]. Although novel immunosuppressive strategies have improved the clinical outcome, the survival of grafts and patients is still poor and the results do not match the success rates of the transplantation of other organs (e.g. kidney, liver or heart). This implies that other, nonimmunological reactions, such as hypoxic damage or ischemia and reperfusion play pivotal roles in the postoperative graft dysfunction and damage [4]. Reperfusion induces a complex phenomenon, including vasoconstriction or leukocyte-endothelial cell interactions, and leads to severe tissue injury over and above the ischemic damage [5–7]. Since the mucosa is the most sensitive part of the intestinal structure, mucosal barrier disruption quickly leads to bacterial and/or endotoxin translocation and septic complications not only after SBTX but also in other cases of occlusive or nonocclusive mesenteric ischemia [4].

Mihály Boros MD, PhD, DSc
Institute of Surgical Research, University of Szeged
PO Box 464
HU–6701 Szeged (Hungary)
Tel. +36 62 545 103, Fax +36 62 545 743, E-Mail boros@expsur.szote.u-szeged.hu

The aim to attenuate ischemia-reperfusion (I/R) injury is as old as the recognition of the phenomenon. Various methods have been proposed and some of them have been applied in clinical practice. Ischemic preconditioning (IPC) was first described by Murry et al. [8] in 1986 and it has subsequently been regarded as a promising tool against I/R in many organs. IPC is the phenomenon that short periods of ischemia make the tissues resistant to the deleterious effects of subsequent, prolonged ischemia. It is divided into 2 phases. The acute, transient reaction occurs within minutes ('classic preconditioning'), involves the upregulation of pre-existing defense mechanisms and is presumably independent of de novo protein synthesis. The other, delayed part ('second window of protection') requires hours for complete induction, is sustained and may require de novo protein synthesis. The underlying mechanism has been studied mainly in the myocardium, but sufficient data concerning the mechanism of IPC in other organs, including the small bowel, are still not available. Recently, it has been shown that the IPC-induced amelioration of I/R injury may involve protection of the villous microcirculation in rodents [9]. Although the above line of reasoning suggests that an improved microcirculation could be closely associated with a better tissue homeostasis during SBTX, the relative contributions of IPC-caused microvascular responses in this setting are unclear.

Our present goal was an adequate description of the posttransplant microcirculatory changes in a large animal model of SBTX. To this aim, the inflammatory cellular reactions and perfusion changes in dogs were characterized separately by means of conventional fluorescence intravital microscopy and an orthogonal polarization spectral (OPS) imaging technique, respectively. Secondly, the therapeutic potential of IPC was investigated during the early reperfusion phase of SBTX.

Materials and Methods

The experiments were performed in adherence to the NIH guidelines for the use of experimental animals. The study was approved by the Ethics Committee for the Protection of Animals in Scientific Research at Szent-Györgyi Albert Medical Center, University of Szeged.

Surgical Preparation

Three series of experiments were performed on a total of 15 inbred dogs (Beagle Kennel of the Animal House of the University of Szeged, average weight 15.4 kg) under sodium pentobarbital anesthesia (30 mg/kg i.v.). The animals were deprived of food, but not water, for 24 h prior to the experiments. Endotracheal in-

tubation was applied to facilitate respiration, and the body temperature was maintained at 37°C with a homeothermic blanket. In aseptic techniques, the right femoral artery and vein were cannulated for the measurement of mean arterial pressure (MAP) and for fluid and drug administration, respectively. All animals received a continuous infusion of Ringer's lactate at a rate of 10 ml/kg/h. A Swan-Ganz thermodilution catheter (Corodyn TD-E-N, 5011-110-7Fr; Braun Melsungen AG, Melsungen, Germany) was placed into the pulmonary artery via the left femoral vein, to measure cardiac output by thermodilution.

In group 1 (n = 5), orthotopic SBTX was performed. Following an upper transverse laparotomy, the spleen was removed, and the root of the superior mesenteric artery (SMA) and vein was dissected free. The mesentery of the small bowel was divided between ligations in the distal (ileocolic) and proximal (duodeno-jejunal) parts and the intestinal wall was transected. The SMA was then cannulated with a 7-F polyethylene catheter and the small intestine was removed from the abdominal cavity. The intestinal graft was perfused with 500 ml chilled (4°C) heparinized Ringer's lactate at a perfusion pressure of 100 cm water. The graft was stored on ice until the vessels were reanastomosed end-to-end with 6/0 running sutures (nonabsorbable polypropylene). After 60-min cold ischemia, reperfusion was induced and the macro- and microhemodynamic changes were observed hourly for 4 h after revascularization.

In group 2 (n = 5), the experimental protocol was identical to that described above, except that an IPC was performed (3 times 5-min SMA occlusion, followed by 10 min of reperfusion) 60 min prior to ischemia induction.

Group 3 (n = 5), served as sham-operated control, without bowel harvesting. In these animals laparotomy and splenectomy were performed and the mesenteric vessels were cannulated for hemodynamic measurements. All manipulations were identical to those in the other, treated groups, with the exception that the bowel was not perfused and harvested. There was no significant difference between the 3 groups as concerns the duration of the surgical preparation or the cold ischemia time in groups 1 and 2.

Hemodynamic Measurements

The central venous pressure and MAP were measured with Statham P23Db transducers. The SMA blood flow (SMAF) was monitored continuously, using an ultrasonic flow probe (Transonic Systems Inc., Ithaca, N.Y., USA) placed around the exposed SMA. Hemodynamic data were registered with a computerized data acquisition system (SPEL Adv Haemosys 2.46, Experimetria Ltd., Budapest, Hungary). The cardiac index (CI) was determined by a thermodilution technique, using a Cardiostar CO-100 computer (Experimetria Ltd.). Arterial blood gases were regularly measured with a blood gas analyzer (AVL, Graz, Austria).

Intravital Fluorescence Videomicroscopy

The mesenteric microcirculation was analyzed by using an epi-illumination technique. An ileal segment with intact neurovascular connections was gently placed on a specially designed stage. During the entire in vivo microscopic procedure, the tissue was constantly bathed in 37°C Ringer's lactate to avoid drying and exposure to ambient air. Leukocyte-endothelial cell interactions in the postcapillary venules of the mesentery were monitored with a high-resolution Zeiss Axiotech Vario 100 HD fluo-
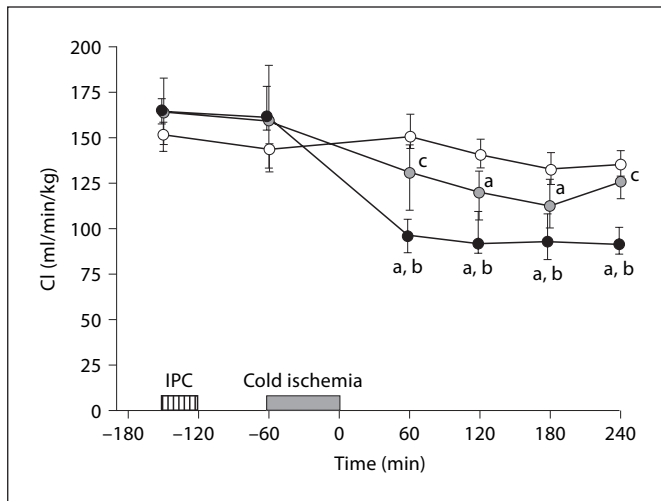
Fig. 1. Changes in CI in the SBTX (black circles, n = 5), preconditioned SBTX (grey circles, n = 5) and sham-operated (open circles, n = 5) groups. Median values and 75th and 25th percentiles are given. [a] p < 0.05 within the groups versus baseline values, [b] p < 0.05 between the sham-operated and SBTX or SBTX + IPC groups, [c] p < 0.05 between SBTX and SBTX + IPC groups.

Fig. 2. Changes in SMA flow in the SBTX (black circles, n = 5), preconditioned SBTX (grey circles, n = 5) and sham-operated (open circles, n = 5) groups. Median values and 75th and 25th percentiles are given. [a] p < 0.05 within the groups versus baseline values, [b] p < 0.05 between the sham-operated and SBTX or SBTX + IPC groups, [c] p < 0.05 between SBTX and SBTX + IPC groups.

rescent intravital microscope (100 W HBO mercury lamp, Acroplan 20 × water immersion objective, Ex: 495 nm, Em: 515 nm). The microscopic images were recorded by a charge-coupled device video camera (AVT HORN-BC 12) attached to an S-VHS video recorder (Panasonic AG-MD 830) and a personal computer. Leukocytes were stained in vivo by means of rhodamine-6G (Mw 479, Sigma, St. Louis, Mo., USA; 0.2%, 0.1 ml i.v.). Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images (Pictron Ltd., Budapest, Hungary) within 5 postcapillary venules per animal, including the observation of nonadherent, adherent and rolling leukocytes. Adherent leukocytes (stickers) were defined in each vessel segment as cells that did not move or detach from the endothelial lining within an observation period of 30 s and are given as the number of cells per square millimeter of endothelial surface, calculated from the diameter and length of the vessel segment observed, assuming cylindrical geometry. Rolling leukocytes were defined as cells moving at a velocity less than 2/5 of that of the erythrocytes in the centerline of the microvessel and are given as a percentage of the nonadherent leukocytes passing through the observed vessel segment within 30 s.

OPS Imaging

An intravital OPS technique (Cytoscan A/R, Cytometrics, Pa., USA) was used to monitor microvascular perfusion changes in the intestinal villi before harvesting and during the reperfusion phase after transplantation. The OPS imaging technique utilizes reflected polarized light at 548 nm, which is the isosbestic point of oxy- and deoxyhemoglobin. Since polarization is preserved in reflection, only photons scattered from relatively deep inside the tissue contribute to the images. In this way, a virtual light source is created in the tissues so that the vessels appear black. The method is
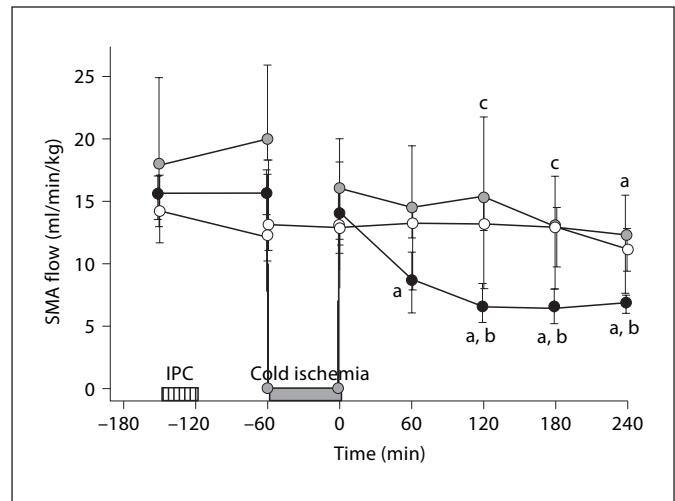
suitable for visualizing all hemoglobin-containing structures, without the use of a fluorescent dye. A 10× objective was introduced into the intestinal lumen through a small enterotomy, on the antimesenteric side of the bowel. The objective was connected to a light source with flexible cables; thus, this intestinal segment was not exteriorized during the study. Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. The functional capillary density (length of perfused nutritive capillaries per observation area (centimeter^{-1}), the red blood cell velocity (millimeters/second) in the central arterioles and venules of the villi and the thickness of the epithelial cell layer covering the intestinal villi (micrometers) were determined in 5 separate fields by means of a computer-assisted image analysis system (IVM Pictron®). All data were expressed as the means of 5 measurements at each time point.

Experimental Protocol

The animals were randomly assigned to one or other of the experimental groups. After a stabilization period, the basal cardiovascular parameters were measured for 20 min. Control OPS measurements (functional capillary density, epithelial thickness and red blood cell velocity) were performed before perfusion. For technical reasons (it needs to transport the experimental animal beyond the microscope), baseline IVM measurement were not performed before perfusion. After these measurements in group 2, complete SMA occlusion was performed 3 times for 5 min, followed by 10 min of reperfusion (IPC). Sixty minutes after IPC, perfusion of the graft was performed, and after 60-min cold ischemia vascular anastomoses were performed, and reperfusion was monitored for 4 h. Leukocyte-endothelial cell interactions were monitored hourly by IVM during the 4-hour reperfusion phase.
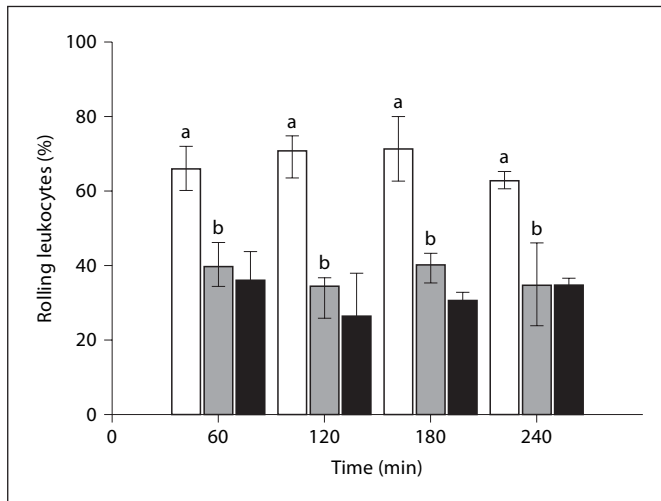
Fig. 3. Percentage of rolling leukocytes in the mesenteric postcapillary venules in the SBTX (black columns, n = 5), preconditioned SBTX (grey columns, n = 5) and sham-operated (white columns, n = 5) groups. The tops of the columns show the median values. The capped bars indicate the 25th and 75th percentiles, respectively. [a] p < 0.05 between the sham-operated and SBTX or SBTX + IPC groups, [b] p < 0.05 between SBTX and SBTX + IPC groups.
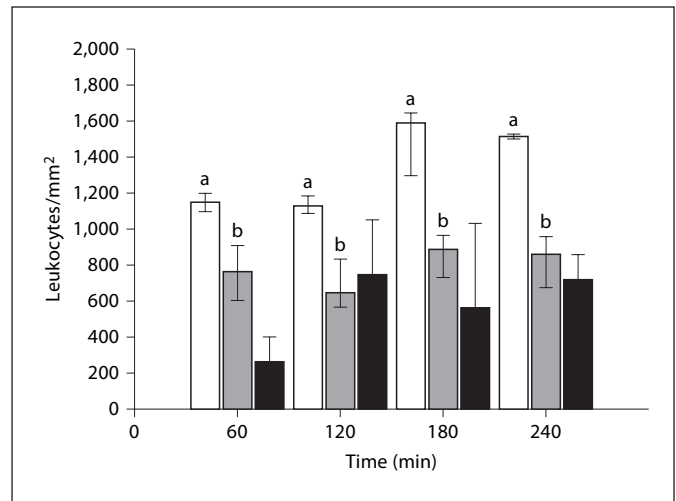

Fig. 4. Number of adherent leukocytes in the mesenteric postcapillary venules in the SBTX (black columns, n = 5), preconditioned SBTX (grey columns, n = 5) and sham-operated (white columns, n = 5) groups. The tops of the columns show the median values. The capped bars indicate the 25th and 75th percentiles, respectively. [a] p < 0.05 between the sham-operated and SBTX or SBTX + IPC groups, [b] p < 0.05 between SBTX and SBTX + IPC groups.

Statistical Analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Nonparametric methods were used. Friedman repeated measures analysis of variance on ranks was applied within the groups. Time-dependent differences from the baseline were assessed by Dunn's method. Differences between groups were analyzed with the Kruskal-Wallis and Dunn's test. In the figures, median values and 75th and 25th percentiles are given. p values <0.05 were considered significant.

Results

Macrohemodynamic Data

MAP decreased significantly during the reperfusion phase, and there were no differences in this parameter between groups 1 and 2. Similarly, a significant 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 (fig. 1). SMAF also decreased during the early phase of reperfusion as compared with the preharvesting level. In the SBTX group the flow remained significantly lower throughout the whole period of reperfusion, while IPC stabilized SMAF on a significantly higher level (fig. 2).

Microcirculatory Parameters 1 (IVM)

For technical reasons, IVM observations could not be performed before graft harvesting, and the baseline data for this parameter are therefore missing. The amount of leukocyte-endothelial cell interactions was significantly higher after SBTX than in the sham-operated group 3. 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 (fig. 3) and the number of firmly adhered white blood cells too (fig. 4).

Microcirculatory Parameters 2 (OPS)

SBTX and reperfusion induced a slight decrease in red blood cell velocity 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 functional capillary density, was significantly decreased during reperfusion in the SBTX group, while this parameter sta-
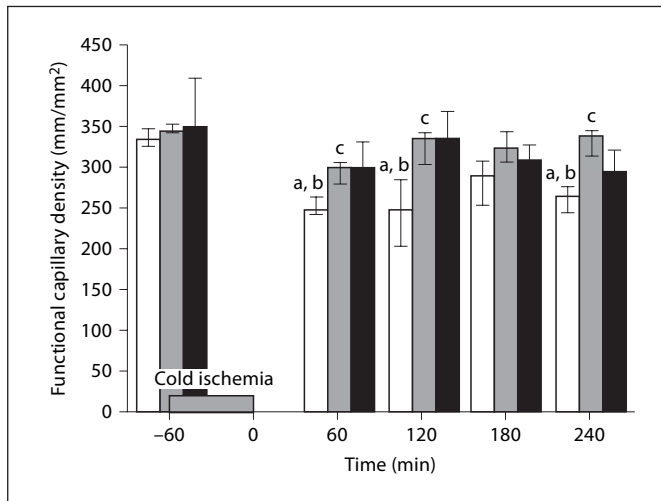
Fig. 5. Changes in functional capillary density in the SBTX (black columns, n = 5), preconditioned SBTX (grey columns, n = 5) and sham-operated (white columns, n = 5) groups. The tops of the columns show the median values. The capped bars indicate the 25th and 75th percentiles, respectively. [a] p < 0.05 within groups versus baseline values, [b] p < 0.05 between the sham-operated and SBTX or SBTX + IPC groups, [c] p < 0.05 between SBTX and SBTX + IPC groups.

Fig. 6. Epithelial thickness of the small intestinal villi in the SBTX (black columns, n = 5), preconditioned SBTX (grey columns, n = 5) and sham-operated (white columns, n = 5) groups. The tops of the columns show the median values. The capped bars indicate the 25th and 75th percentiles, respectively. [a] p < 0.05 within groups versus baseline values, [b] p < 0.05 between the sham-operated and SBTX or SBTX + IPC groups, [c] p < 0.05 between SBTX and SBTX + IPC groups.

bilized at close to the control level in the SBTX + IPC group (fig. 5).

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 group was reduced at 60 min relative to baseline, but thereafter it was unchanged, suggesting less severe epithelial structural damage (fig. 6).

Discussion

This study provides the first description of the microcirculatory consequences of IPC before 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 with autotransplantation to mimic clinical conditions but to exclude possible immunological complications. Accordingly, these observations relate to clear I/R-related changes only.
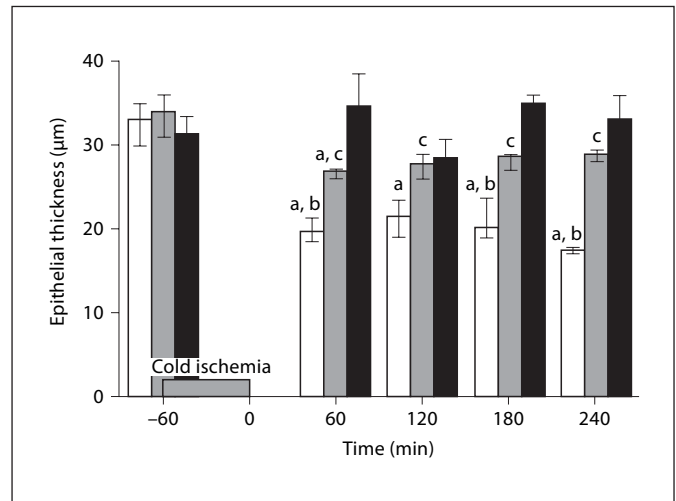
Reperfusion of the intestinal graft was accompanied by significant macrocirculatory deterioration. The mesenteric blood flow decreased and the concomitantly falling CI remained lower during the 4 h of reperfusion. The CI is mainly determined by the heart rate and cardiac stroke volume. Since there was no considerable change in heart rate or cardiac preload (central venous pressure) or afterload (MAP), a depressed contractile function of the heart may have contributed to the reduced CI. This might suggest that myocardial depressant substrates were liberated during reperfusion from the previously ischemic mesenteric vascular bed [10]. The severe decrease in SMAF may, at least in part, be a consequence of the reduced CI, but the role of local mesenteric circulatory reactions cannot be ruled out. IPC stabilized both the SMAF and the CI near the control, preharvesting levels. The beneficial effect on the CI suggests that intestinal IPC may exert some distant effect on the myocardial function, probably by reducing the ischemic injury of the bowel, resulting in a lower extent of myocardial depressant release [10].

In addition to systemic hemodynamic changes, significant alterations were observed in the microcirculatory system of the mesenteric vascular bed. A considerable capillary perfusion failure evolved, in parallel with

significant leukocyte-endothelial cell interactions. IPC, when applied before graft perfusion, improved the microcirculation and afforded protection from epithelial injury. Our results are in accordance with those of Mallick et al. [9], who demonstrated an improved capillary perfusion and a reduced amount of leukocyte-endothelial cell interactions following IPC in a rodent model of warm ischemia and reperfusion. We earlier observed similar changes in the same model and underlined the important role of endogenously liberated endothelin-1 in the pathogenesis of these changes [11]. Similar effects were observed on the exogenous infusion of endothelin-1 [12]. It has been demonstrated that IPC protects the reperfused endothelium by reducing the endothelin-1-induced activation of NADPH oxidase and xanthine oxidase in the rat heart [13]. On the other hand, the tissue microcirculation is regulated by different vasoactive mediators in both normal and pathologic conditions. With the suggestion of the important role of the vasoconstrictor and proadhesive endothelin during reperfusion, the protective effect of IPC may be mediated by counterbalancing this effect. The consequence of I/R is that the endothelium is characterized by decreased nitric-oxide (NO)-dependent relaxation. NO is one of the most potent endogenous vasodilators and antiadhesive mediators [14] and the role of NO has previously been demonstrated as a potential mechanism of IPC in the intestine [15]. NO has been suspected as an important factor in the adaptive processes following preconditioning both in the heart and in the intestine [15–17]. Recent studies suggest that NO could have a bifunctional role, as a trigger [18] and mediator in classic IPC [19]. IPC may be a trigger for the constitutively active intestinal endothelial and/or neuronal NO synthase, and NO is able to counterbalance the vasoconstrictive effects of endothelin and other vasoactive mediators at the precapillary level. On the other hand, NO can also act as an antiadhesive mediator, limiting leukocyte-endothelial cell interactions and leukocyte-mediated tissue injury.

The exact roles of NO and the different isoforms of NO synthase in IPC are not yet understood, however, it seems that the endothelial isoform is involved in early protection, while both endothelial and inducible NO synthase isotypes play roles in delayed consequences of IPC [20, 21].

The development of leukocyte-endothelial cell interactions requires the expression of adhesion molecules on the surface of leukocytes and endothelial cells. It has been reported that I/R induces a 7-fold increase in P-selectin expression in the rat jejunum, and IPC significantly decreases this expression. These data indicate that P-selec-

tin expression is a novel downstream effector target of the anti-inflammatory signaling pathway in IPC [22].

Probably all of the above-mentioned protective mechanisms may have contributed to the beneficial microcirculatory effects of IPC in our experiments, but we have to stress that this study focused on the early changes only. Nevertheless, the vascular endothelium of the reperfused tissue could be one of the most important target of IPC, and the prevention of endothelial dysfunction may result in reduced structural tissue injury. Although we did not perform histological investigations, the villi were directly observed with intravital microscopy, and the height and appearance of epithelial cell layer suggested that significant tissue protection was induced by IPC.

Given the essential role of the endothelium and NO in the regulation of the vascular tone and the platelet and leukocyte functions, the protection of endothelial cells is an important therapeutic target. In this context, a number of studies have shown that both early and delayed preconditioning may prevent an endothelial dysfunction after I/R. This endothelial protection most probably results from the inhibitory effects of preconditioning on the expression of endothelial adhesion molecules, leading to reduced neutrophil-endothelial interactions. The mechanisms of early endothelial preconditioning may involve mediators such as adenosine, bradykinin, NO and free radicals, together with the activation of protein kinase C and the opening of ATP-sensitive potassium channels. With regard to delayed preconditioning, recent studies have shown that both NO and free radicals are involved as triggers of this second window of endothelial protection. The complex interactions between these 2 radical species ultimately lead to a delayed increase in NO production, which is most probably responsible for the decreased adhesion of neutrophils to endothelial cells [23].

Additionally to the microcirculatory failure developing during reperfusion, reactive oxygen free radicals generated during reperfusion of the hypoxic tissues or synthesized by activated leukocytes also play a pivotal role in the tissue injury [24] and there is evidence that both early and late IPC reduces the reperfusion-linked oxidative stress in the small intestine [25, 26]. It has been proposed that superoxide radicals play an important role in the signaling pathway of IPC [27]. Reactive hyperemia induced by short ischemic episodes provides the short-lived protection of early-phase IPC through a xanthine-oxidase-dependent increase in superoxide production [28]. A recent experimental study by our group demonstrated a reperfusion-induced significant increase in tissue xanthine oxidase activity and a marked elevation in superoxide production at the begin-

ning of reperfusion. However, in the preconditioned animals, no significant change was observed at the beginning of reperfusion [29, 30]. All these data suggest that IPC exerts its beneficial effect by limiting free radical-mediated tissue injury during reperfusion.

In conclusion, our study has demonstrated for the first time the beneficial microcirculatory effects of IPC during the early phase of reperfusion following SBTX in a large animal model. The exact mechanisms of this considerable tissue-protective effect of IPC need further investigation, especially as concerns the direction of potential pharmacological preconditioning, but the promising results suggest that IPC could be an optional method with which to reduce reperfusion injury in cases when bowel ischemia is predictable.

References

1 Langnas AN: Advances in small intestine transplantation. Transplantation 2004;77: S75–S78.
2 Reyes J, Bueno J, Kocoshis S, Green M, Abu-Elmagd K, Furukawa H, Barksdale EM, Strom S, Fung JJ, Todo S, Irish W, Starzl TE: Current status of intestinal transplantation in children. J Pediatr Surg 1998;33:243–254.
3 Grant D: Current results of intestinal transplantation. Lancet 1996;347:1801–1803.
4 Mallick IH, Yang W, Winslet MC, Seifalian AM: Ischemia-reperfusion injury of the intestine and protective strategies against injury. Dig Dis Sci 2004;49:1359–1377.
5 Massberg S, Gonzalez AP, Leiderer R, Menger MD, Messmer K: In vivo assessment of the cold preservation time on microvascular reperfusion injury after experimental small bowel transplantation. Br J Surg 1998; 85:127–133.
6 Gonzalez AP, Sepulveda S, Massberg S, Baumeister R, Menger MD: In vivo fluorescence microscopy for the assessment of microvascular reperfusion injury in small bowel transplants in rats. Transplantation 1994;58: 403–408.
7 Schoenberg MH, Poch B, Younes M, Schwarz A, Baczako K, Lundberg C, Haglund U, Beger HG: Involvement of neutrophils in postischaemic damage to the small intestine. Gut 1991;32:905–912.
8 Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986;74:1124–1136.
9 Mallick IH, Yang W, Winsletand MC, Seifalian AM: Protective effects of ischemic preconditioning on the intestinal mucosal microcirculation following ischemia-reperfusion of the intestine. Microcirculation 2005;12:615–625.
10 Haglund U: Systemic mediators released from the gut in critical illness. Crit Care Med 1993;21(Suppl 2):S15–S18.
11 Wolfard A, Szalay L, Kaszaki J, Sahin-Toth G, Vangel R, Balogh A, Boros M: Dynamic in vivo observation of villus microcirculation during small bowel autotransplantation: effects of endothelin-A receptor inhibition. Transplantation 2002;73:1511–1513.
12 Boros M, Massberg S, Baranyi L, Okada H, Messmer K: Endothelin 1 induces leukocyte adhesion in submucosal venules of the rat small intestine. Gastroenterology 1998;114: 103–114.
13 Duda M, Konior A, Klemenska E, Beresewicz A: Preconditioning protects endothelium by preventing ET-1-induced activation of NADPH oxidase and xanthine oxidase in post-ischemic heart. J Mol Cell Cardiol 2007; 42:400–410.
14 Rubanyi GM, Polokoff MA: Endothelins: molecular biology, biochemistry, pharmacology, physiology and pathophysiology. Pharmacol Rev 1994;46:325–415.
15 Hotter G, Closa D, Prados M, Fernandez-Cruz L, Prats N, Gelpi E, Rosello-Catafau J: Intestinal preconditioning is mediated by a transient increase in nitric oxide. Biochem Biophys Res Commun 1996;222:27–32.
16 Vegh A, Szekeres L, Parratt J: Preconditioning of the ischaemic myocardium; involvement of the L-arginine nitric oxide pathway. Br J Pharmacol 1992;107:648–652.
17 Sola A, De Oca J, Gonzalez R, Prats N, Rosello-Catafau J, Gelpi E, Jaurrieta E, Hotter G: Protective effect of ischemic preconditioning on cold preservation and reperfusion injury associated with rat intestinal transplantation. Ann Surg 2001;234:98–106.
18 Lochner A, Marais E, Du Toit E, Moolman J: Nitric oxide triggers classic ischemic preconditioning. Ann NY Acad Sci 2002;962:402–414.
19 Xuan Yt, Tang Xl, Qiu Y, Banerjee S, Takano H, Han H, Bolli R: Biphasic response of cardiac NO synthase isoforms to ischemic preconditioning in conscious rabbits. Am J Physiol 2000;279:H2360–H2371.
20 Cutrin JC, Perrelli MG, Cavalieri B, Peralta C, Catafau JR, Poli G: Microvascular dysfunction induced by reperfusion injury and protective effect of ischemic preconditioning. Free Rad Biol Med 2002;33:1200–1208.
21 Huang SS, Wei FC, Hung LM: Ischemic preconditioning attenuates postischemic leukocyte-endothelial cell interactions – role of nitric oxide and protein kinase C. Circ J 2006;70:1070–1075.
22 Davis JM, Gute DC, Jones S, Krsmanovic A, Korthuis RJ: Ischemic preconditioning prevents postischemic P-selectin expression in the rat small intestine. Am J Physiol 1999; 277:H2476–H2481.
23 Laude K, Beauchamp P, Thuillez C, Richard V: Endothelial protective effects of preconditioning. Cardiovasc Res 2002;55:466–473.
24 Granger DN, Rutili G, McCord JM: Superoxide radicals in feline intestinal ischemia. Gastroenterology 1981;81:22–29.
25 Ferencz A, Racz B, Cserepes B, Roth E: The effects of classic and delayed ischemic preconditioning on the oxidative stress in small bowel autotransplantation model. Magy Seb 2005;58:245–249.
26 Ferencz A, Szanto Z, Borsiczky B, Kiss K, Kalmar-Nagy K, Szeberenyi J, Horvath PO, Roth E: The effects of preconditioning on the oxidative stress in small-bowel autotransplantation. Surgery 2002;132:877–884.
27 Tritto I, Ambrosio G: Role of oxidants in the signaling pathway of preconditioning. Antioxid Redox Signal 2001;3:3–10.
28 Peralta C, Bulbena O, Xaus C, Prats N, Cutrin JC, Poli G, Gelpi E, Rosello-Catafau J: Ischemic preconditioning: a defense mechanism against the reactive oxygen species generated after hepatic ischemia reperfusion. Transplantation 2002;73:1203–1211.
29 Horvatics G, Hajdú N, Juhász T, Kaszaki J, Torday CS, Boros M: The role of mast cells in the mechanism of intestinal ischaemic preconditioning. Acta Physiol Hung 2004;91: 307–308.
30 Kaszaki J, Wolfárd A, Szalay L, Boros M: Pathophysiology of ischemia-reperfusion injury. Transplant Proc 2006;38:826–828.

II. Varga S, Juhász L, Gál P, Bogáts G, Boros M, Palásthy Z, Szabó A, Kaszaki J. Neuronal nitric oxide mediates the anti-inflammatory effects of intestinal ischemic preconditioning. J Surg Res. 2019;244:241-250.

Check for updates

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

Sándor Varga, MD,[a] László Juhász, PhD,[b] Péter Gál, MD,[c] Gábor Bogáts, MD, PhD,[a] Mihály Boros, MD, DSc,[b] Zsolt Palásthy, MD, PhD,[d] Andrea Szabó, MD, PhD,[b,] and József Kaszaki, PhD[b]*

[a] *Department of Cardiac Surgery, Faculty of Medicine, University of Szeged, Szeged, Hungary*
[b] *Institute of Surgical Research, Faculty of Medicine, University of Szeged, Szeged, Hungary*
[c] *Department of Pediatrics, Faculty of Medicine, University of Szeged, Szeged, Hungary*
[d] *Department of Surgery, Faculty of Medicine, University of Szeged, Szeged, Hungary*

ARTICLE INFO

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.

© 2019 Published by Elsevier Inc.

* *Corresponding author.* Institute of Surgical Research, University of Szeged, Pulz u. 1, H-6724 Szeged, Hungary. Tel.: +36-62 545-103; fax: +36-62 545-743.
E-mail address: szabo.andrea.exp@med.u-szeged.hu (A. Szabó).

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 intestine[4] involves reduced activation of transcription factor nuclear factor-kappa binding[5] 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 reperfusion[8] 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 dogs[12] 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 rodents[10] 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 IκBα 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 pCO_2
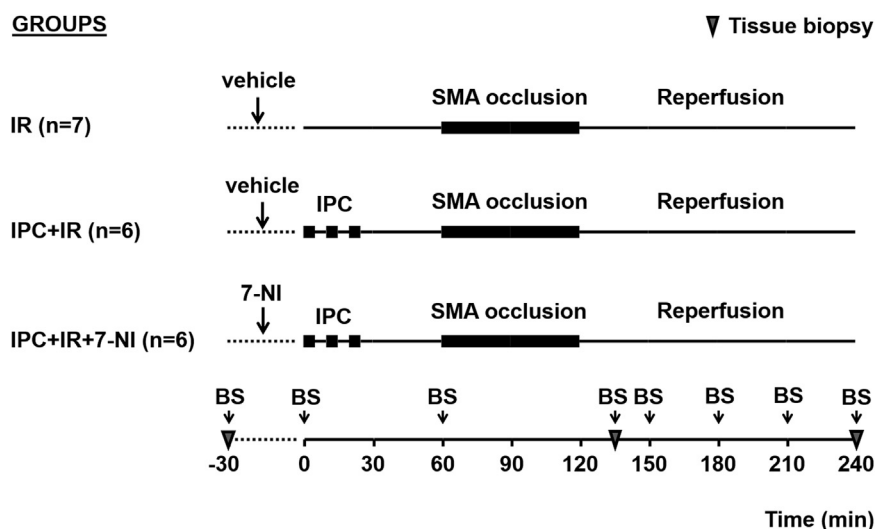
Fig. 1 – Experimental protocol. In the first group, 60 min of ischemia was induced by occluding the SMA, which was followed by 120 min of reperfusion (IR). In the two other groups, IR was preceded by IPC, which was elicited by inducing three cycles of 5-min ischemia/5-min reperfusion periods in the presence 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 Ca^{2+}-dependent and Ca^{2+}-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 μm), and stained with Alcian blue and Safranin O. Histologic analysis was performed in coded sections by one investigator (S.V.) at 40× 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× 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× 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
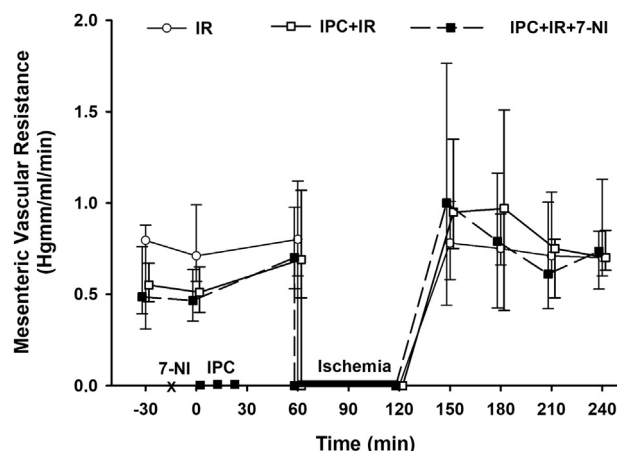

Fig. 2 – Time course of changes in mesenteric vascular resistance during baseline and during 60 min of SMA occlusion followed by 120 min of reperfusion (IR group). Further groups of animals were also subjected to IPC in the presence and absence of 7-NI treatment (IPC + IR+7-NI and IPC + IR groups, respectively). 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

Ca^{2+}-independent and Ca^{2+}-dependent NOS activities were not significantly different between examined groups at baseline (Fig. 6A and B). As for Ca^{2+}-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, 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 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.
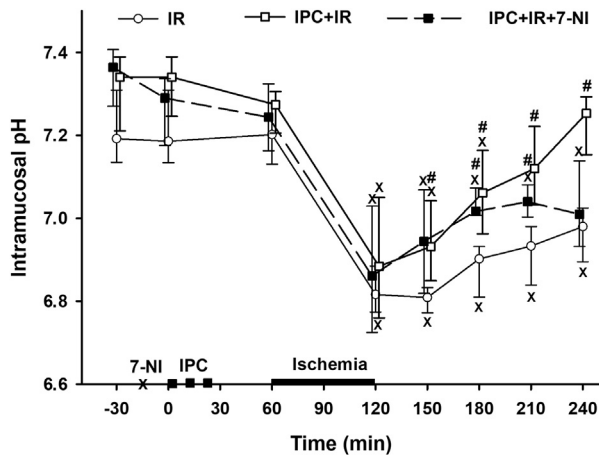
Fig. 3 — Time course of changes in intramucosal pH during baseline and 60 min of SMA occlusion followed by 120 min of reperfusion (IR group). Further groups of animals were also subjected to IPC in the presence and absence of 7-NI treatment (IPC + IR+7-NI and IPC + IR groups, respectively). Here, values 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.
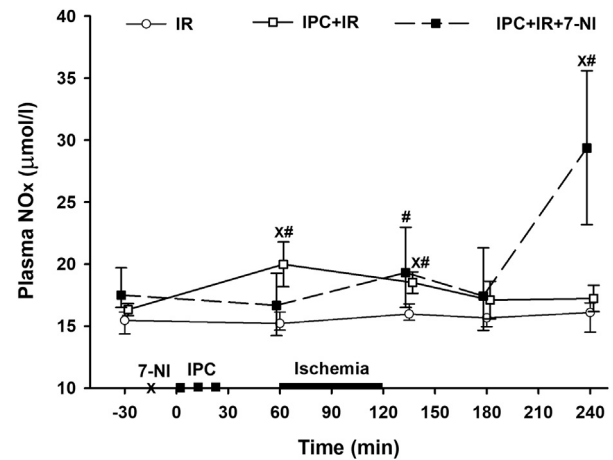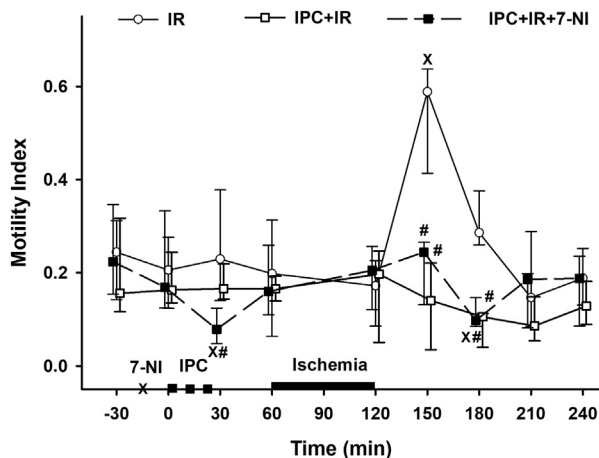

Fig. 5 — Time course of changes in plasma NO_x during baseline and during 120 min of reperfusion after a 60-min of SMA occlusion (IR group). Further groups of animals were also subjected to IPC in the presence and absence of 7-NI treatment (IPC + IR+7-NI and IPC + IR groups, respectively). Here, values 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]
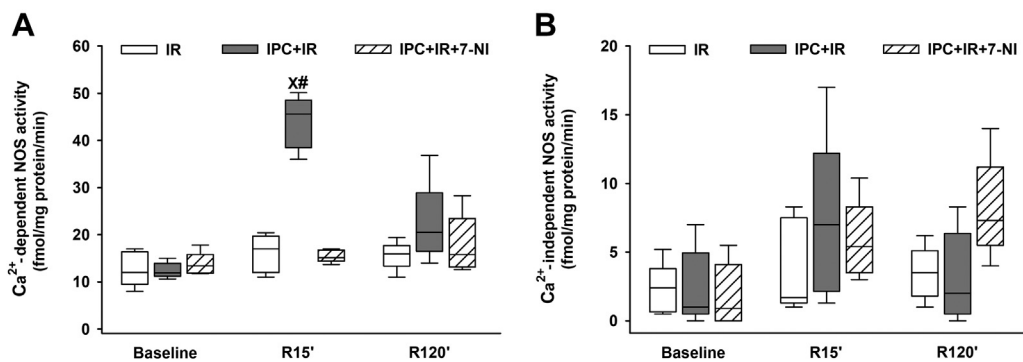
Fig. 6 — Changes in (A) constitutive NO synthase (Ca^{2+}-dependent NOS) and (B) inducible NO synthase (Ca^{2+}-independent NOS) activities in the affected ileal segment during baseline and at 15 and 120 min of reperfusion (IR). Further groups of animals were also subjected to IPC in the presence and 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

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.

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 reperfusion[31] 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 NO[33] 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
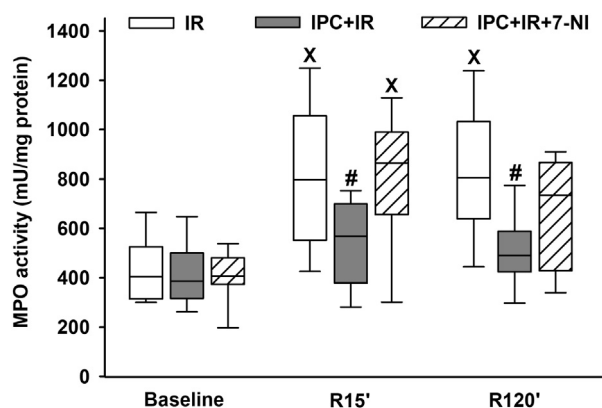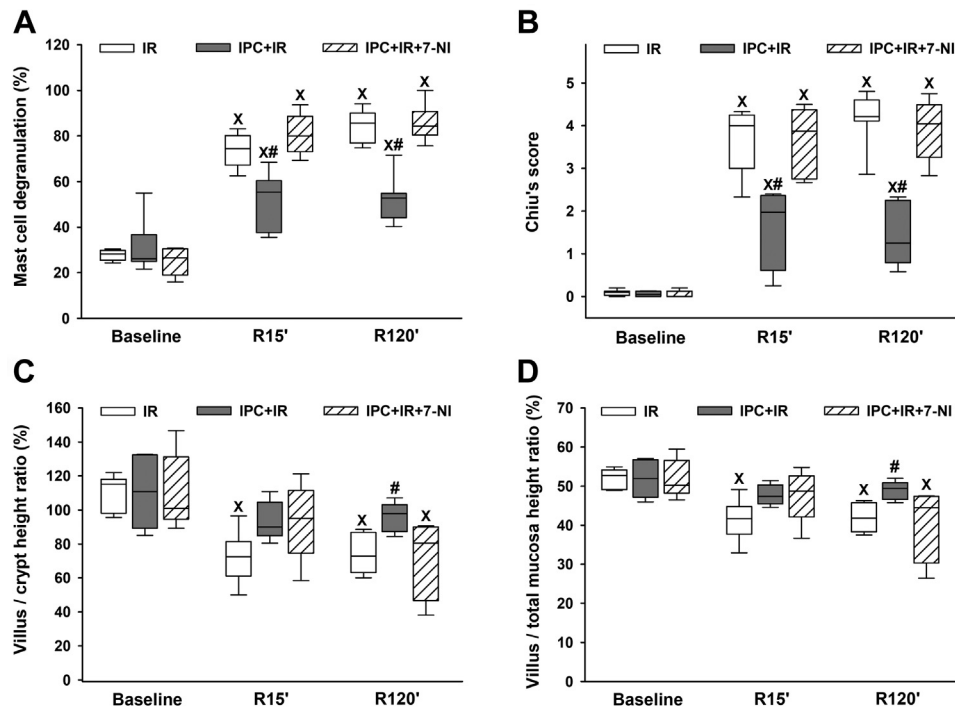
Fig. 8 – Changes in (A) MC degranulation, (B) mucosal histologic injury according to Chiu's grade, and mucosal height ([C] as expressed as villus/crypt height ratio and [D] villus/total mucosa height ratio) in samples taken from the affected ileal segment during baseline and at 15 and 120 min of reperfusion (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 poly-morphonuclear 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 inhibi-tion.[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 pre-vented 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 inflam-matory reactions (e.g., polymorphonuclear leukocyte accu-mulation and MC degranulation); these can also be inhibited by IPC.[43,44] We saw simultaneous early increases in both tis-sue MPO and MC degranulation as early as 15 min after ischemia, which were both ameliorated by IPC. Similar, rela-tively early MPO changes were also demonstrated in rats (examined 30-60 min after ischemia).[41,43] The nNOS de-pendency of the latter reaction was also proven as inhibition of nNOS (or KO)[36] reversed this protection. Interestingly, the
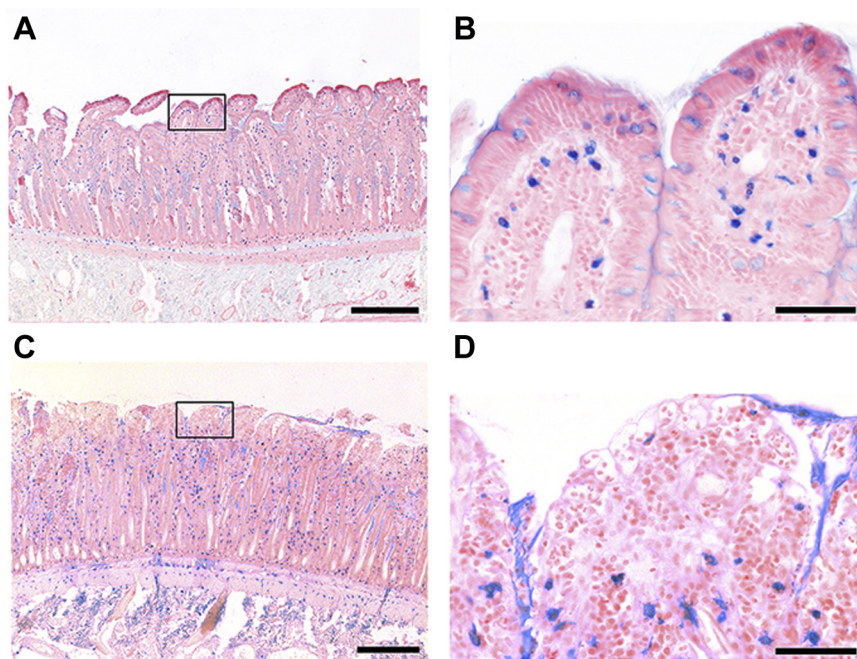
Fig. 9 — Representative micrographs showing mucosal MCs stained with Alcian blue and Safranin O at (A and C) lower (bar denotes 200 μm) and (B and D) higher magnifications (bar denotes 50 μm). In Panel B, please note intact intracellular granules, whereas Panel D shows a loss of intracellular granules 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.)
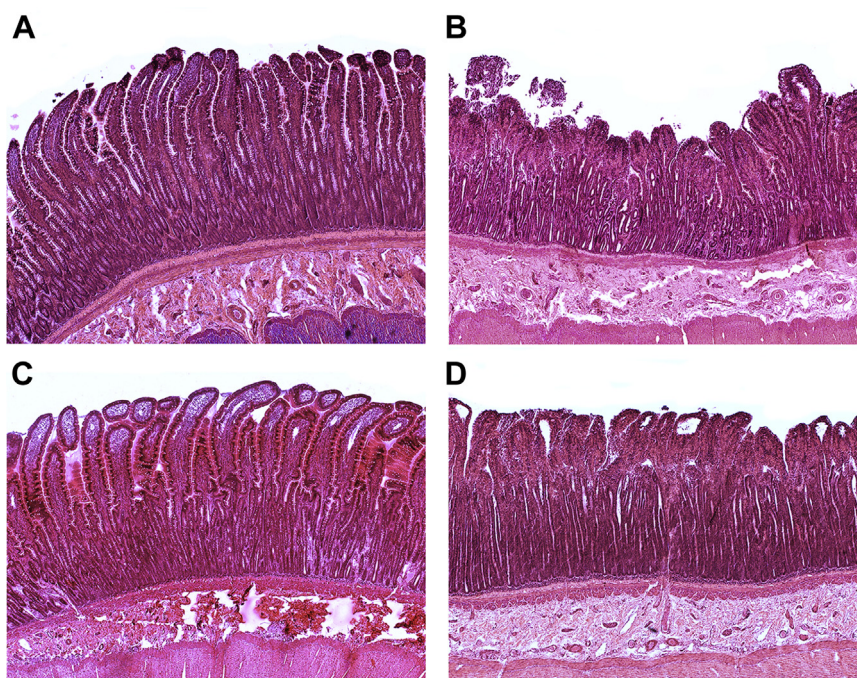

Fig. 10 — Representative micrographs of the ileal mucosa stained with hematoxylin and eosin staining at (A) baseline and (B) 120 min of reperfusion in the IR group, (C) the IPC + IR group, and (D) the IPC + IR+7-NI group. Panels B and D demonstrate massive epithelial lifting down the side of villi, denuded 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 μm 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 macro-hemodynamic 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.

Acknowledgment

Disclosure

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

REFERENCES

1. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation*. 1986;74:1124−1136.
2. Hotter G, Closa D, Prados M, et al. Intestinal preconditioning is mediated by a transient increase in nitric oxide. *Biochem Biophys Res Commun*. 1996;222:27−32.
3. Wolfárd A, Kaszaki J, Varga S, Lázár G, Boros M. Early microcirculatory changes after ischemic preconditioning and small bowel autotransplantation. *Eur Surg Res*. 2007;39:284−290.
4. Camprodon RA, Bowles MJ, Pockley AG, de Oca J. Anti-inflammatory effects of ischemic preconditioning on rat small bowel allografts. *Transplant Proc*. 2014;46:2146−2149.
5. Ferencz A, Szántó Z, Borsiczky B, et al. The effects of preconditioning on the oxidative stress in small-bowel autotransplantation. *Surgery*. 2002;132:877−884.
6. Sileri P, Sica G, Gentileschi P, et al. Ischemic preconditioning protects intestine from prolonged ischemia. *Transplant Proc*. 2004;36:283−285.
7. Taha MO, Miranda-Ferreira R, Chang AC, et al. Effect of ischemic preconditioning on injuries caused by ischemia and reperfusion in rat intestine. *Transplant Proc*. 2012;44:2304−2308.
8. Calcina F, Barocelli E, Bertoni S, et al. Effect of N-methyl-d-aspartate receptor blockade on neuronal plasticity and gastrointestinal transit delay induced by ischemia/reperfusion in rats. *Neuroscience*. 2005;134:39−49.
9. Kubes P. Ischemia-reperfusion in feline small intestine: a role for nitric oxide. *Am J Physiol*. 1993;264:G143−G149.
10. Qu XW, Wang H, Rozenfeld RA, Huang W, Hsueh W. Type I nitric oxide synthase (NOS) is the predominant NOS in rat small intestine. Regulation by platelet-activating factor. *Biochim Biophys Acta*. 1999;1451:211−217.
11. Kanwar S, Tepperman BL, Payne D, Sutherland LR, Kubes P. Time course of nitric oxide production and epithelial dysfunction during ischemia/reperfusion of the feline small intestine. *Circ Shock*. 1994;42:135−140.
12. Kawata K, Takeyoshi I, Iwanami K, et al. A spontaneous nitric oxide donor ameliorates small bowel ischemia-reperfusion injury in dogs. *Dig Dis Sci*. 2001;46:1748−1756.
13. Palásthy Z, Kaszaki J, Lázár G, Nagy S, Boros M. Intestinal nitric oxide synthase activity changes during experimental colon obstruction. *Scand J Gastroenterol*. 2006;41:910−918.
14. Qu XW, Thaete LG, Rozenfeld RA, et al. Tetrahydrobiopterin prevents platelet-activating factor-induced intestinal hypoperfusion and necrosis: role of neuronal nitric oxide synthase. *Crit Care Med*. 2005;33:1050−1056.
15. Iijima H, Tulic MK, Duguet A, et al. NOS 1 is required for allergen-induced expression of NOS 2 in mice. *Int Arch Allergy Immunol*. 2005;138:40−50.
16. Altay T, Gonzales ER, Park TS, Gidday JM. Cerebrovascular inflammation after brief episodic hypoxia: modulation by neuronal and endothelial nitric oxide synthase. *J Appl Physiol (1985)*. 2004;96:1223−1230.

17. Beck PL, Xavier R, Wong J, et al. Paradoxical roles of different nitric oxide synthase isoforms in colonic injury. *Am J Physiol Gastrointest Liver Physiol*. 2004;286:G137–G147.

18. Qu XW, Wang H, De Plaen IG, Rozenfeld RA, Hsueh W. Neuronal nitric oxide synthase (NOS) regulates the expression of inducible NOS in rat small intestine via modulation of nuclear factor kappa B. *FASEB J*. 2001;15:439–446.

19. Furness JB, Callaghan BP, Rivera LR, Cho HJ. The enteric nervous system and gastrointestinal innervation: integrated local and central control. *Adv Exp Med Biol*. 2014;817:39–71.

20. Moore PK, Bland-Ward PA. 7-nitroindazole: an inhibitor of nitric oxide synthase. *Methods Enzymol*. 1996;268:393–398.

21. Huge A, Kreis ME, Jehle EC, et al. A model to investigate postoperative ileus with strain gauge transducers in awake rats. *J Surg Res*. 1998;74:112–118.

22. Kuebler WM, Abels C, Schuerer L, Goetz AE. Measurement of neutrophil content in brain and lung tissue by a modified myeloperoxidase assay. *Int J Microcirc Clin Exp*. 1996;16:89–97.

23. Gera L, Varga R, Török L, et al. Beneficial effects of phosphatidylcholine during hindlimb reperfusion. *J Surg Res*. 2007;139:45–50.

24. Chiu CJ, McArdle AH, Brown R, Scott HJ, Gurd FN. Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. *Arch Surg*. 1970;101:478–483.

25. McCallion K, Wattanasirichaigoon S, Gardiner KR, Fink MP. Ischemic preconditioning ameliorates ischemia- and reperfusion-induced intestinal epithelial hyperpermeability in rats. *Shock*. 2000;14:429–434.

26. Yang B, Chen Y, Long YH, et al. Intestinal and limb ischemic preconditioning provides a combined protective effect in the late phase, but not in the early phase, against intestinal injury induced by intestinal ischemia-reperfusion in rats. *Shock*. 2018;49:596–603.

27. Pontell L, Sharma P, Rivera LR, et al. Damaging effects of ischemia/reperfusion on intestinal muscle. *Cell Tissue Res*. 2011;343:411–419.

28. Ji YY, Wang ZD, Wang SF, et al. Ischemic preconditioning ameliorates intestinal injury induced by ischemia-reperfusion in rats. *World J Gastroenterol*. 2015;21:8081–8088.

29. Sola A, De Oca J, González R, et al. Protective effect of ischemic preconditioning on cold preservation and reperfusion injury associated with rat intestinal transplantation. *Ann Surg*. 2001;234:98–106.

30. Watanabe T, Owada S, Kobayashi H, et al. Real-time monitoring of nitric oxide (NO) and pO2 levels under ischemic conditions associated with small bowel ischemia/reperfusion injury using selective electrodes for NO and oxygen molecules. *Transplant Proc*. 2007;39:3007–3009.

31. Lai CH, Lee CH, Hung CY, Lo HC. Oral citrulline mitigates inflammation and jejunal damage via the inactivation of neuronal nitric oxide synthase and nuclear factor-κB in intestinal ischemia and reperfusion. *JPEN J Parenter Enteral Nutr*. 2017;41:422–435.

32. Giaroni C, Marchet S, Carpanese E, et al. Role of neuronal and inducible nitric oxide synthases in the Guinea pig ileum myenteric plexus during in vitro ischemia and reperfusion. *Neurogastroenterol Motil*. 2013;25:e114–e126.

33. Zweier JL, Wang P, Samouilov A, Kuppusamy P. Enzyme-independent formation of nitric oxide in biological tissues. *Nat Med*. 1995;1:804–809.

34. Barua A, Standen NB, Galiñanes M. Dual role of nNOS in ischemic injury and preconditioning. *BMC Physiol*. 2010;10:15.

35. Lu XM, Zhang GX, Yu YQ, et al. The opposite roles of nNOS in cardiac ischemia-reperfusion-induced injury and in ischemia preconditioning-induced cardioprotection in mice. *J Physiol Sci*. 2009;59:253–262.

36. Rivera LR, Pontell L, Cho HJ, et al. Knock out of neuronal nitric oxide synthase exacerbates intestinal ischemia/reperfusion injury in mice. *Cell Tissue Res*. 2012;349:565–576.

37. Palombit K, Mendes CE, Tavares-de-Lima W, Silveira MP, Castelucci P. Effects of ischemia and reperfusion on subpopulations of rat enteric neurons expressing the P2X7 receptor. *Dig Dis Sci*. 2013;58:3429–3439.

38. Rivera LR, Thacker M, Pontell L, Cho HJ, Furness JB. Deleterious effects of intestinal ischemia/reperfusion injury in the mouse enteric nervous system are associated with protein nitrosylation. *Cell Tissue Res*. 2011;344:111–123.

39. Castro M, Muñoz JM, Arruebo MP, et al. Involvement of neuronal nitric oxide synthase (nNOS) in the regulation of migrating motor complex (MMC) in sheep. *Vet J*. 2012;192:352–358.

40. Gil V, Gallego D, Grasa L, Martín MT, Jiménez M. Purinergic and nitrergic neuromuscular transmission mediates spontaneous neuronal activity in the rat colon. *Am J Physiol Gastrointest Liver Physiol*. 2010;299:G158–G169.

41. Filpa V, Carpanese E, Marchet S, et al. Nitric oxide regulates homeoprotein OTX1 and OTX2 expression in the rat myenteric plexus after intestinal ischemia-reperfusion injury. *Am J Physiol Gastrointest Liver Physiol*. 2017;312:G374–G389.

42. Moore-Olufemi SD, Kozar RA, Moore FA, et al. Ischemic preconditioning protects against gut dysfunction and mucosal injury after ischemia/reperfusion injury. *Shock*. 2005;23:258–263.

43. de Oca J, Hotter G, Sola A, et al. Role of nitric oxide in preconditioning for intestinal transplantation. *Transplant Proc*. 1999;31:2573.

44. Xing D, Zhang R, Li S, et al. Pivotal role of mast cell carboxypeptidase A in mediating protection against small intestinal ischemia-reperfusion injury in rats after ischemic preconditioning. *J Surg Res*. 2014;192:177–186.

45. Jansson L, Carlsson PO, Bodin B, Andersson A, Källskog O. Neuronal nitric oxide synthase and splanchnic blood flow in anaesthetized rats. *Acta Physiol Scand*. 2005;183:257–262.

46. Mallick IH, Yang W, Winslet MC, Seifalian AM. Ischaemic preconditioning improves microvascular perfusion and oxygenation following reperfusion injury of the intestine. *Br J Surg*. 2005;92:1169–1176.

47. Mallick IH, Yang W, Winslet MC, Seifalian AM. Protective effects of ischemic preconditioning on the intestinal mucosal microcirculation following ischemia-reperfusion of the intestine. *Microcirculation*. 2005;12:615–625.