ROLE OF ENDOTHELIN IN THE DEVELOPMENT OF MESENTERIC LOW-FLOW STATES

Ph.D. Dissertation

László Szalay M.D.

Institute of Surgical Research
Szent-Györgyi Albert Medical and Pharmaceutical Center
University of Szeged
Hungary

2003

CONTENTS

LIST OF PAPERS RELATED TO THE SUBJECT OF THE DISSERTATION

I. László Szalay, József Kaszaki, Sándor Nagy and Mihály Boros: THE ROLE OF ENDOTHELIN-1 IN CIRCULATORY CHANGES DURING HYPODYNAMIC SEPSIS IN THE RAT. *Shock* 10(2):123-128, 1998.

II. Antal Wolfárd, László Szalay, József Kaszaki, Gábor Sahin-Tóth, Róbert Vangel, Ádám Balogh and Mihály Boros: DYNAMIC IN VIVO OBSERVATION OF VILLUS MICROCIRCULATION DURING SMALL BOWEL AUTOTRANS-PLANTATION: EFFECTS OF ENDOTHELIN-A RECEPTOR INHIBITION. *Transplantation* 73(9):1511-1513, 2002.

III. László Szalay, József Kaszaki, Sándor Nagy and Mihály Boros: ENDOTHELIN-1 INDUCES MUCOSAL MAST CELL DEGRANULATION IN THE RAT SMALL INTESTINE. *Life Sciences* 67(16):1947-1958, 2000.

IV. Mihály Boros, László Szalay and József Kaszaki: ENDOTHELIN-1 INDUCES MUCOSAL MAST CELL DEGRANULATION AND TISSUE INJURY VIA ET-A RECEPTORS. *Clinical Science (London)*103(S48):31S-34S, 2002.

SUMMARY

Intestinal injury resulting from acute ischemia is a significant life-threatening event. Following the reduction or blockade of the nutritive blood flow, structural and functional damage develops. Reperfusion, however, also leads to a sequence of events, characterized by vasoconstriction, endothelial swelling and leukocyte activation. There is a growing body of evidence, that the release of endothelium-derived, potent vasoactive peptides, endothelins (ETs), plays a crucial role in the development of ischemia-reperfusion processes. ETs can mediate not only long-lasting vasoconstriction, but contribute to the induction of leukocyte and mast cell activation as well.

Leukocyte-endothelial cell interactions are a decisive factor in the sequelae of ischemia-reperfusion injuries; while adherent leukocytes on the endothelial surface may obstruct microcirculation the extravasated ones, producing oxygen free radicals and further cytotoxic agents can cause other tissue injuries.

Intestinal mast cells (MCs) are a cellular source of stored, preformed, and *de novo* synthesized inflammatory mediators which could be involved in further reactions induced by ischemia. Although several observations suggest the importance of MCs in the ET-mediated circulatory disorders, their role in this process is still not completely understood. Our aim was to examine the interference among ETs, mast cell and leukocyte activation in the ischemic intestine. Therefore, in the first part of the study, ET-mediated leukocyte-endothelial cell interactions were examined in a clinically relevant ischemia-reperfusion model. With this background, in the second set of experiments, the consequences of exogenously administered endothelin-1 (ET-1) on mast cell degranulation and leukocyte activation were studied.

Following intestinal ischemia-reperfusion, the ET-A receptor antagonist ETR-P1/fl peptide significantly reduced the endogenous ET-mediated leukocyte activation with a simultaneous attenuation of hemodynamic and morphological impairments. In the second part of the study, exogenous ET-1 was infused to mimic endogenous ET release. In these groups, both ET-A receptor antagonists and MC membrane stabilizer compound sodium-cromoglycate mitigated ET-1-induced intestinal MC degranulation and leukocyte activation simultaneously. In conclusion, our data indicate an interaction among ET-release, MC and leukocyte activation in the intestine. The amelioration of the functional and structural impairments following either an ET-A receptor antagonist or a mast cell stabilizer agent suggests a potential new therapeutical regimen in ischemia-related pathophysiologic states of the gastrointestinal tract.

1. INTRODUCTION

1.1. The ischemia-reperfusion syndrome

Tissue injury, following ischemia is not only the consequence of the hypoxic insult, but also due to reperfusion resulting in an inflammatory response (Granger 1988, Parks 1986). After the ischemic event, during the early phase of reperfusion the sequential release of free radicals, cytokines and vasoactive mediators leads to the activation of both endothelial and inflammatory cells. The evolving circulatory state during reperfusion can be characterized by vasoconstriction, free radical generation, and granulocyte activation (Eppihimer 1997). Although the main source of free radicals is the xanthine/xanthine oxidase system, the infiltrating neutrophils are also potential sources of reactive oxygen species and cytokine release as well (Granger 1988, Lojek 1997, Schoenberg 1991). Thus, beyond vasoconstriction, the oxidative burst, capillary plugging, endothelial cell swelling and interstitial edema induced by recruited leukocytes also exacerbate the impairment of microcirculation (Boros 1995).

1.1.1. *Endothelins*

Endothelin was originally described as the most potent vasoconstrictor peptide produced by endothelial cells. Under physiologic conditions ET is produced predominantly by the endothelium, but in pathophysiological states other cells, such as leukocytes, macrophages, smooth muscle cells, cardiomyocytes and mesangial cells can also be the source of ET release (Sessa 1991, Ehrenreich 1990, Hahn 1990, Ito 1993, Fukunaga 1991).

Endothelin release is regulated by both rheological and chemical factors such as pulsatile stretch, shear stress and pH (MacArthur 1994, Malek 1992, Wesson 1998). Hypoxia is considered one of the basic stimuli for ET synthesis (Rakugi 1990). Cytokines, adhesion molecules or vasoacting agents also stimulate ET production (Imai 1992, Bodin 1995).

ET isoforms are coded by at least three distinct genes; following transcription ETs are formed by multiple cleavage via prepro-ET and big-ET then. The process may be significantly influenced by the activity of ET converting enzyme (ECE) isoforms, present in endothelial, smooth muscle cells, cardiomyocytes and macrophages. Due to the functional and structural similarities with neutral endopeptidases, ECE independent mechanisms may also contribute to ET production (Turner 1997). The process results 21-residue peptide isoforms of

ET-1, ET-2, and ET-3. The responsiveness to ET isopeptides is heterogenous in a variety of vascular and nonvascular tissues.

Once formed, the physiological and pathophysiological actions of ETs are mediated by at least two distinct receptor subtypes, named ET-A and ET-B, both of them from the family of G-protein-coupled receptors. The ET-A receptor has a higher affinity for ET-1 and ET-2 than ET-3, whereas the ET-B receptor has an equal affinity for all isopeptides (Arai 1990, Sakurai 1990). The receptors mediate different circulatory effects depending on their localization, but it is suggested that vasoconstriction is mediated predominantly via the ET-A subtype, while the activation of ET-B receptors elicits both vasodilator and vasoconstrictor responses (Clozel 1992, Sumner 1992, Shetty 1993).

In the vasculature ET-A receptors localized on smooth muscle cells, whereas ET-B_1 receptors are found on endothelial, and to a lesser extent, ET-B_2 in smooth muscle cells. ET-A receptors have an exclusive affinity the ET-1 peptide, but ET-B receptors are without any isoform preference. Stimulation of ET-A and ETB_2 receptors leads to an activation of phospholipase C, with a subsequent accumulation of inositol triphosphate and intracellular calcium, resulting in vasoconstriction. In contrast, ET-B_1 receptors mediate the release of nitric-oxide and prostacyclin and inhibit ECE expression in the endothelium. ET-B receptors also involved in the clearance of the circulating ET as well (Fukuroda 1994, Ozaki 1995).

Beyond other functions not listed here, ET is involved in bronchoconstriction (Uchida 1998) and contributes in inflammation processes either by stimulating cytokine production or by the direct induction of mast cell degranulation, neutrophil adhesion or platelet aggregation (Agui 1994, Hofman 1998, Ninomiya 1992, Yamamura 1994, Elferink 1994, Boros 1998, Espinosa 1996). The role of ET in these processes is supported by the fact, that elevated plasma or tissue ET levels were registered in certain cardiovascular diseases and asthma.

1.1.2. *ET receptor antagonists*

Specific blockade of ET receptors is a promising tool for the elucidation of the role of the ETs in physiologic regulation and in different pathologic conditions. Responding to this demand, numerous ET receptor antagonists have been developed in the past decade. While some of these are non-selective, and inhibit both ET-A and ET-B receptors (i.e. Bosentan, Gardiner 1994), other antagonists have specificity toward either the ET-A receptor (BQ-123, BQ-610, Ihara 1992, Ishikawa 1992) or the ET-B receptor subtype (IRL-1038, Karaki 1993). Due to the controversial effects, mediated by ET-B receptors a specific antagonist against the ET-B_1 receptor subtype has also been developed (RES-701-1, Tanaka 1994).

The ETR-P1/fl peptide is an antisense homology box-derived peptide, with strong inhibitory potency against the ET-A receptor. The ETR-P1/fl peptide was developed by using the antisense homology boxes of the human ET-A receptor. The sense-antisense interaction means that the peptide synthesized from the inactive DNA strand, is complementary to the peptide translated from the active DNA strand. Therefore peptides could recognize and bind to each other (Blalock 1984). Moreover, a sense-antisense interaction exists not only between individual peptides, but also within two parts of the one peptide chain. These amino acid sequences are termed antisense homology boxes (Baranyi 1995).

In previous *in vitro* experiments, ETR-P1/fl peptide induced relaxation in ET-1-preconstricted vessel rings. The reference drug, a commercially available, highly specific ET-A receptor antagonist BQ-123 exerted the same effect in this setting, demonstrating the ET-A receptor-inhibitory property of the ETR-P1/fl peptide (Baranyi 1998).

1.1.3. *Mast cells*

Mast cells are derived from multipotential stem cells in bone marrow. Mast cell precursors are also found in bone marrow, blood, mucosal and connective tissues. When appropriate stimulation is given, the precursors proliferate and differentiate into mast cells (Kitamura 1993). The wide tissue distribution of mast cells and their ability to dramatically increase in number in several pathologic conditions has led to an evidence about their functions, including regulation of allergic inflammation, gastric acid secretion, and circulation. Mast cells contain special cytoplasmic granules which store mediators of inflammation. The extracellular release of the mediators is known as *degranulation* and may be induced by physical destruction, toxins, endogenous mediators or immune mechanisms. There are two main categories of inflammatory (anaphylactic) mediators in mast cells. The preformed mediators, stored in secretory granules and secreted upon cell activation, include biogenic amines, histamine, proteoglycans, and/or heparin, over-sulphate chondroitin sulphates, and a wide range of neutral proteases. The typical mediator, histamine acts at H_1, H_2 and H_3 receptors on cells and tissues, modulating smooth muscle contraction, nitric oxide formation, vascular permeability, neurotransmitter release as well. Proteoglycan has two functions: on the one hand it packages histamine and basic proteins into secretory granules, and on the other hand it can regulate the stability of the protease called tryptase. Neutral proteases, which account for the vast majority of the granule protein, serve as markers of different types of mast cells. Some other mediators, often absent in the resting mast cells, are typically released after *de novo* synthesis, principally leukotriene C_4, prostaglandin D_2 and

cytokines including TNF-α, IL-3, IL-4, IL-6, IL-8 (Galli, 1993, Lin 1993). Furthermore, the observation that MCs produce ETs and express ET receptors suggests that ETs may modulate MC function even in an autocrine manner too (Ehrenreich 1992, Yamamura 1994).

In a series of important studies, Enerbäck on the basis of histochemical and fixation characteristics demonstrated that intestinal mucosal mast cells differ from connective tissue mast cells (Enerbäck 1989). Mast cells from various anatomic sites differ in their content of mediators such as neutral proteases, as well as in their response to various secretagogues and anti-allergic compounds.

Human mast cell heterogeneity is also characterized by histochemical, ultrastructural, and functional differences between cell populations (Galli 1993, Irani 1994, Welle 1997). Two main types have been divided; those mast cells which contain both tryptase and chymase named MC^{TC} while those with tryptase but not chymase were classified in the MC^{T} subtype. Although there are some similarities, human mast cell populations are not analogous in all respects to rodent intestinal and connective tissue MCs. Like rats and mice, human mast cell populations differ in their content of granule-associated neutral proteases, albeit in ways distinct from rodent mast cells. However the anatomic location of MC^{T} and MC^{TC} populations is not as distinct as with rodent mucosal and connective tissue MCs; while the MC^{T} predominates in the intestinal mucosa and lung, MC^{TC} type is dominant in the normal skin and intestinal submucosa.

Due to the great number of mediators and their effector mechanisms, the inferences of MCs in the regulatory processes might be controversial.

1.1.4. *Leukocyte-endothelial cell interactions*

Whereas several mechanisms have been identified in the development and progression of reperfusion injury, one of the main contributor of the pathogenesis is leukocyte-mediated tissue dysfunction and injury. At the beginning of this process, activated endothelial cells produce chemoattractants such as platelet activating factor, leukotriene B4 and chemokines on their luminar surface. A major chemokine, IL-8 is released upon stimulation of IL-1, LPS, histamine or thrombin. The chemoattractants then may initiate firm leukocyte adhesion, chemotaxic movement. Oxidative burst or degranulation, usually are completed at a latter phase of activation.

The process when leukocytes recognize and transiently interact with the vascular endothelium is characterized as leukocyte "rolling". The rolling step is mediated by neutrophil L-selectin and by the newly expressed E- and P-selectins on inflamed endothelial cells. While

P-selectin, expressed within minutes on the endothelial cell surface following stimulation by thrombin, histamine, or oxygen radicals, E-selectin appears on the endothelial surface one or two hours after cell stimulation by IL-1, TNF-α, or LPS. Unlike P- and E-selectins, L-selectin is constitutively present on leukocytes. Its binding capacity is however rapidly increased after leukocyte activation, possibly via receptor oligomerization (Li 1998). The ligands for the selectins are carbohydrate-like structures present on the surface of endothelial cells and leukocytes.

The next step is the strengthening of adhesive forces, which results in the capture and firm adherence of leukocytes to the endothelium. In this phase leukocytes shed their L-selectin and increase their expression of $ß_2$ integrins (CD11a,b,c/CD18). The leukocyte integrins engage ligands on the endothelial cells to develop a strong adhesive interaction. The major ligand for $ß_2$ integrins is intercellular adhesion molecule (ICAM-1), which is constitutively expressed on the endothelium, and is up-regulated in response to cytokine stimulation.

Finally, neutrophil transmigration occurs prominently at the borders of endothelial cells via tight junction discontinuities. The target for neutrophils is the P-selectin, localized along the endothelial borders (Burns 1999). Extravasation requires concomitant modifications of endothelial the intercellular junctions as well. Neutrophils migrate then towards their final target mediated by ß1-ß3 integrins.

1.2. The intestinal tract in low-flow states

1.2.1. *Endothelins in mesenteric low-flow states*

Increased release of ET under ischemic conditions is well-documented in different circulatory regions (Firth 1992, Brunner 1995, Ferri 1995). Elevated plasma levels were measured following superior mesenteric artery occlusion (Schlichting 1995). Causing additional vasoconstriction the released ET may exacerbate local hypoxia. Additionally, the gut circulation appears to be more susceptible to the vasoconstrictor response to ET-1 than any non-gastrointestinal tissues in the rat (Maclean 1989). The sustained and significant vasoconstriction during the reperfusion phase demonstrates the importance of factors in the local milieu. Reactive oxygen species may also contribute to the increased sensitivity. ET-1 may enhances the free radical production of prestimulated granulocytes (Ishida 1990). On the other hand, the reduced plasma ET-1 concentration following superoxide dismutase pretreatment raises the possibility of an additional ET production (Oktar 2002). It has been

also shown that reactive oxygen species generated from hypoxanthine/xanthine oxidase caused an increased ET receptor expression in the liver (Gabriel 1998).

It has been reported that the administration of exogenous ET-1 induces leukocyte activation; a key element of the development of ischemia-reperfusion processes (Boros 1998). Mast cells of the intestinal tract, can also be activated by ET challenge. Activation of different subtypes of MCs has been reported (Yamamura 1994, Egger 1995, Liu 1998).

Similarly, the attenuation of ischemia-reperfusion injury of the intestine following ET receptor antagonist application suggests a decisive role for ETs in the gastrointestinal tract (Mitsuoka 1999, Oktar 2002).

1.2.2. *Mast cells in mesenteric ischemia-reperfusion*

There is a growing body of evidence that MC may contribute to ischemia-reperfusion injury in the reperfused tissues. Activation of MCs during ischemia-reperfusion has been proved in many circulatory regions (Boros 1989, Masini 1990, Goldman 1992. Galli 1993). MC degranulation has been demonstrated histologically in the intestinal tract of the rat (Kurose 1994, Kanwar 1994). Release of the major mediator of MC, histamine, was also reported following intestinal ischemia-reperfusion (Boros 1991). Inhibition of MC degranulation resulted in the amelioration of ischemia-reperfusion injuries not only in the intestine but in the lung and heart as well (Boros 1995, Vural 2000, Frangogiannis 1998). Reactive oxygen species generated from the early phase of reperfusion might play a prominent role in MC activation; these are not just causing histamine release, but are generated by MCs as well (Boros 1991, Kanwar 1994).

Production and expression of both ETs and their receptors have been demonstrated in different populations of mast cells (Ehrenreich 1992, Yamamura 1994). Additionally, MC-derived chymases can enhance ET-formation via the cleavage of big-ET to the active form (Wypij 1992). Thus ET release can also potentiate MC activation under ischemic conditions.

Moreover, it has been recognized that MCs release many potent neutrophil chemoattractants, which can recruit leukocytes, thus MCs contribute to the development of tissue injury via leukocyte activation (Galli 1993, Gaboury 1995).

Despite the similarities in the underlying mechanisms, the magnitude of MC degranulation in the ischemia-reperfusion injury could be significantly different in various tissues. One possible explanation for the high degranulatory activity in gastrointestinal tissue is the high number of MCs in this circulatory region. MC subtypes also can be a plausible source of the differences: their variable sensitivities to the activating agents and their different

mediator contents may lead to disparate reactions. Furthermore, the proximity to the intramural neural plexus provides an additional organ-specific environment. Considering its sensitivity and effector potential, neuronal regulation also could be a definitive regulator of the MC activation. Finally, the loose tissue character of the intestinal mucosa subserves edema formation, thus histamine release from MCs may cause microcirculatory impairment directly.

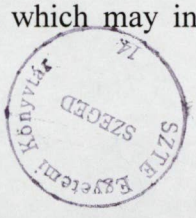
1.2.3. *Common examples of mesenteric ischemia in clinical practice; small bowel transplantation*

Mesenteric ischemia or ischemia-reperfusion is involved in several pathologic conditions. Since the mesenteric circulation utilizes a relatively large part of the cardiac output, circulatory disturbances affecting this area often lead to severe local tissue injury and systemic hemodynamic reactions. Any pathophysiological state related to depressed cardiac output leads to the redistribution of the circulation, and (as part of this process) mesenteric vasoconstriction evolves, leading to low-flow ischemia.

Local obstruction of the blood flow either at the arterial (embolism, thrombosis and hernia incarceration) or the venous side of the vasculature induces mesenteric ischemia. The intestinal mucosa, which is a metabolically active, hypoxia-sensitive tissue, forms a barrier between the non-sterile intestinal lumen and the sterile circulation. Therefore the loss of restrictive mucosal function leads to a transmural movement of toxic factors into the circulation, causing sepsis and possibly multiple organ failure (Bahrami 1997).

A large number of diseases can lead to an insufficient absorption capacity of the intestine. Two-thirds of all patients are infants with congenital malformations (intestinal atresia or microvillus inclusion bodies), causing malabsorption, but some other diseases (radiation enteritis, Gardner's syndrome and Crohn's disease) appearing in children and adults (Frezza 1996) may also cause this abnormality. Transplantation of the small intestine now offers an optional treatment modality in these conditions (Kocoshis 1994). The application of this therapy is limited by the high rate of immunologic and non-immunologic complications (Grant 1996, Reyes 1998). However immunologic complications are attenuated by the application of novel immunosuppressant strategies, but patient survival rate is still only 50%, and the graft survival rate is also lower than after the transplantation of other organs (Grant 1996, Rossi 1996).

Beyond immunologic responses, ischemia-reperfusion injury of the transplanted intestine is the main pathologic agent which may influence the development of the early



microcirculatory complications of the intestinal graft (Pirenne 1988). The appearance of this phenomena is common, even if ischemia and reperfusion are well controlled during transplantation procedures (Cryer 1997). Its significance is evident in the light of the fact, that the majority of patients die of septic complications (Grant 1996), which can be induced by bacterial or endotoxin translocation, probably as a result of a hypoxia induced mucosal injury (Spada 1996, Browne 1991). Albeit rejection episodes also contribute to the impairment of the intestinal barrier, the development of ischemia-reperfusion injury seems to be also decisive in the success of small bowel transplantation.

2. AIMS

There is a growing body of evidence, that ETs play a crucial role in the development of ischemia-reperfusion injury. Their role includes not only mediating long-lasting vasoconstriction, but induction of inflammatory cells as well. The activated leukocytes, mast cells and other sources of inflammatory mediators then can modify the development of ischemia-reperfusion states significantly.

Thus, our aims were to

- *to examine the contribution of ETs to the leukocyte-endothelial cell interactions and circulatory impairment in a clinically relevant intestinal ischemia-reperfusion model, and*
- *to investigate the supposed crosslink between the ET-induced mast cell and leukocyte activation in the rat intestine.*

In **Study I,** following exogenous ET-1 infusion, we determined the contribution of ET-A and B receptors in the ET-1-mediated systemic hemodynamic alterations and controlled the *in vivo* effectiveness of the ET-A receptor antagonist ETR-P1/fl peptide.

In **Study II,** we investigated the role of ET-A receptors in the mesenteric macro- and microcirculatory parameters, leukocyte-endothelial cell interactions following small bowel autotransplantation.

In **Study III,** the influence of ET-A and ET-B receptors on the exogenous ET-1-induced MC degranulation, microcirculatory changes and tissue damage were examined in the rat small intestine.

In **Study IV,** we compared the effects of MC stabilization and ET-A receptor blockade on the ET-1-induced alterations in macrohemodynamics, intestinal histamine release and granulocyte accumulation in a rat model.

3. MATERIALS AND METHODS

3.1. Surgical procedure

3.1.1. Study I. Instrumentation for the measurement of systemic hemodynamics

38 male Wistar rats (weighing 250±15 g) were deprived of food, but not water, for 12 h prior to the experiments. The animals were anesthetized with sodium pentobarbital (60mg/kg body weight i.p.) and placed in a supine position on a heating pad, and tracheotomy was performed. The right jugular vein was cannulated with PE50 tubing for fluid administration. The left common carotid artery was exposed, and a thermistor-tip catheter was advanced into the ascending aorta to measure CO. The right femoral artery was cannulated with PE50 tubing for mean arterial pressure (MAP) and heart rate (HR) measurements.

3.1.2. Study II. Small bowel autotransplantation

Experiments were performed on a total of 15 mongrel dogs (weighing 15.4±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. The trachea was intubated to facilitate respiration and the body temperature was maintained at 37 °C with a homeothermic blanket. Using aseptic techniques, the right femoral artery and vein were cannulated for the measurement of 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.

In groups 1 and 2 orthotopic small bowel transplantation (SBTX) was performed. Following an upper transverse laparotomy, the spleen was removed, and the root of the superior mesenteric artery (SMA) was dissected free. An ultrasonic flow-probe (Transonic Systems Inc., Ithaca, NY, USA) was placed around the exposed vessel to measure mesenteric blood flow. A branch of the ileocolic artery and a tributary of the ileal vein supplying the terminal part of the ileum were cannulated with 2-F polyethylene catheters to measure the mesenteric arterial and venous pressures, respectively. The mesentery was divided between ligations at the distal (ileo-colic) and proximal (duodeno-jejunal) part of the small bowel, and the intestinal wall was transected. SMA was then cannulated with a 7-F polyethylene catheter and the intestinal graft was perfused with 500 mL of chilled (4 °C) heparinized Ringer's lactate at 100 water-cm perfusion pressure. The cold ischemia time was 60 min. The graft was stored on ice until the vessels were reanastomized end-to-end with 6/0 running sutures (non-

absorbable polypropylene). The animals were observed for 4 h after the revascularization, and then animals were killed with an overdose of pentobarbital.

Group 3 served as sham-operated controls to exclude hemodynamic changes related solely to surgery and to the time elapsed under anesthesia. In these experiments, the animals were infused continuously with Ringer's lactate and received the vehicle for ETR-P1/fl peptide (saline) at matching time points after surgery. There was no significant difference between the groups as concerns the duration of the surgical preparation and the cold ischemia time.

3.1.3. Study III. Peripheral cannulation; isolation of an intestinal segment

42 male Sprague-Dawley rats (weighing 200±20 g) were deprived of food but not water, for 12 h prior to the experiments. The animals were anesthetized with sodium pentobarbital (60 mg/kg body weight i.p.) after premedication with atropine (0.1 mg/kg s.c.), and placed in a supine position on a heating pad for maintenance of the body temperature between 36 and 37 °C. Tracheotomy was performed and the animals were mechanically ventilated with a rodent respirator (Harvard Apparatus, South Natick, MA, USA).

Polyethylene catheters (PE 50, ID 0.58 mm, Fa. Portex, Hythe, UK) were inserted into the left carotid artery and jugular vein for the recording of mean arterial pressure and the injection of test compounds, respectively. Throughout the experiment, the animals received an infusion of Ringer's lactate at a rate of 40 ml/kg/h. After a transverse laparotomy, a segment of the terminal ileum perfused by a single artery was selected. The marginal vessels were divided and ligated, and the intestinal segment with intact neurovascular connections was covered by plastic sheets.

3.1.4. Study IV. Instrumentation for the measurement of systemic hemodynamics

Experiments were performed on a total of 28 male Wistar rats (weighing 200 ±20g) Animals were anesthetized with sodium pentobarbital (60mg/kg i.p.) and placed in a supine position on a heating pad, and tracheotomy was performed. The right external jugular vein was cannulated with PE50 tubing for fluid administration and plasma sampling. The left common carotid artery was exposed, and a thermistor-tip catheter was advanced into the ascending aorta to measure CO. The right femoral artery was cannulated with PE50 tubing for MAP and HR measurements.

3.2. Hemodynamic measurements

3.2.1. Blood pressures and heart rate (in Studies I-IV)

MAP (in Studies I-IV) and also superior mesenteric arterial (SMAP) and venous (SMVP) pressures (in Study II) were monitored with Statham P23Db transducers (Hemosys, Experimetria Ltd., Budapest, Hungary). HR was calculated from the blood pressure signal (in Studies I-IV). Data were recorded with a computerized data-acquisition system (Hemosys, Experimetria Ltd., Budapest, Hungary).

3.2.2. Cardiac output and peripheral resistance (in Studies I and IV)

CO was determined by thermodilution, using a Cardiostar CO-100 computer (Experimetria Ltd., Budapest, Hungary). A 0.2 mL aliquot of physiologic saline at room temperature was used as indicator. Measurements were made in duplicate at each sampling time point, and the average value of the two measurements was calculated. Total peripheral vascular resistance index (TPR) was calculated via the formula MAP/CO*body weight.

3.2.3. Superior mesenteric artery flow and mesenteric vascular resistance (in Study II)

Superior mesenteric artery blood flow (SMAF) was monitored continuously with an ultrasonic flow-meter (Transonic Systems Inc., Ithaca, NY, USA). The flow-probe was placed around the root of the SMA. Mesenteric vascular resistance (MVR) was calculated by using the standard formula (SMAP-SMVP)/SMAF*body weight. Hemodynamic data were recorded with a computerized data-acquisition system (Hemosys, Experimetria Ltd., Budapest, Hungary). Arterial blood gases were measured with a blood gas analyser (Radiometer, Copenhagen, Denmark).

3.2.4. Laser-Doppler measurements (in Study III)

Intestinal intramural blood flow of the segment was recorded with a laser-Doppler flow monitor (Moor Instruments, MBF 3D, Axminster, UK). Laser-Doppler flowmetry affords an estimate of the tissue perfusion, based on continuous measurement of the red blood cell (RBC) flux (the number of red blood cells x velocity). At each observation point, 3 measurements were performed over a 1 to 2-min period.

3.3. Intravital video microscopy (in Study II)

3.3.1. Intravital fluorescent video microscopy (IVM)

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 37^0C 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 100HD fluorescent intravital microscope (100 W HBO mercury lamp, Acroplan 20x water immersion objective, Ex: 495nm, Em: 515nm). The microscopic images were recorded by a charge-coupled device video camera (AVT HORN-BC 12) attached to a 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 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 sec, and are given as number of cells per mm^2 of endothelial surface, calculated from diameter and length of vessel segment observed, assuming cylindrical geometry. Rolling leukocytes were defined as cells moving at a velocity less than 2/5 of that of erythrocytes in the centerline of the microvessel, and are given as percentage of nonadherent leukocytes passing through the observed vessel segment within 30 sec.

3.3.2. Orthogonal Polarization Spectral (OPS) imaging

Intravital OPS technique (Cytoscan A/R, Cytometrics, PA, USA) was used for monitoring 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 wavelength, which is the isosbestic point of oxy- and deoxyhemoglobin. Since polarization is preserved in reflection, only photons scattered from relatively deep in tissue contribute to the images. This way, a virtual light source is created in tissues so that 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, at the antimesenteric side of the bowel.

Quantitative assessment of microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. Functional capillary density (FCD, length of perfused nutritive capillaries per observation area (cm^{-1}) red blood cell velocity (RBCV, μm/sec), the diameter of central arterioles of the villi (μm), and the thickness of the epithelial cell layer, covering the intestinal villi (μm) was determined in 5 separate fields by a computer-assisted image analysis system (IVM Pictron®, Budapest, Hungary). Every data was expressed as the mean of 5 measurements at each time point.

3.4. Histology and Light Microscopy (in Study III)

Intestinal biopsy samples for light microscopy were rapidly placed into ice-cold Carnoy's fixative and trimmed along the longitudinal axis. The fixed tissue was attached to a hard cardboard backing to ensure the optimal longitudinal direction of the section. The samples were embedded in paraffin, sectioned (6 μm) and stained with hematoxylin–eosin. Mucosal damage was assessed on the standard scale of Chiu et al. (Chiu 1970). The grading was performed on coded sections with the following criteria: grade 0, normal mucosa; grade 1, development of subepithelial Gruenhagen's space at the tip of the villus; grade 2, extension of the space with moderate epithelial lifting; grade 3, massive epithelial lifting with a few denuded villi; grade 4, denuded villi with exposed capillaries; and grade 5, disintegration of the lamina propria, ulceration and hemorrhage. Five randomly selected non-overlapping fields per section were inspected and the median grade of injury was then calculated.

MCs were stained positively, according to the alcian blue-safranin O (pH 0.4)-staining. MCs were quantitated in the villi of an average of 20 villus-crypt units using coded sections at 400x optical magnification by one investigator. Loss of intracellular granules, stained material dispersed diffusely within the lamina propria was taken as evidence of MC degranulation.

3.5. Biochemical measurements (in Study IV)

3.5.1. Tissue myeloperoxidase activity (MPO)

MPO activity, as a marker of tissue leukocyte infiltration, was measured in ileal biopsies. MPO activity was determined according to the method of Kuebler et al. (Kuebler 1996).

3.5.2. Plasma histamine assay

Blood samples (0.3 ml) were taken into precooled, heparinized Eppendorf tubes, centrifuged (at 4°C, 13000g, for 3 min) and stored at –70 °C until measurements. Histamine assay was performed by means of a slightly modified version of the radioenzymatic method of Beaven and Horakova (Beaven, Horakova 1978). Briefly, 25µl of plasma was added to a reaction mixture of 25 µl histamine-N-methyltransferase and 25µl of 0.1 M phosphate buffer (pH 7.9) which contained 1 µCi of S-adenosyl[^{3}H-methyl]methionine (Amersham, UK; 60-80 Ci/mmol). Histamine-N-methyltransferase, the enzyme that converts histamine to methylhistamine was prepared from the rat kidney (Verburg 1983). The samples were incubated at 37°C for 90 min, and the reaction was stopped by adding 25µl of 2.5 M $NaBO_4$ (pH10). The labelled histamine was then extracted with 10µl of 10 M NaOH and 0.8 ml of chloroform. The radioactivity was determined with a liquid scintillation counter (Tri-Carb Liquid scintillation Analyzer 2100TR/2300TR, Packard Instruments Co, Meriden, CT, USA).

3.5.3. Tissue histamine assay

Tissue samples were taken with the frozen clamp and put immediately into liquid nitrogen and stored at –70°C until analysis. The tissues were then weighed and homogenized in 5x volume of 0.1 M ice-cold phosphate buffer (pH 6.8) using an ultra-turrax potter (T25 b; IKA Labortechnik, Germany). The homogenates were centrifuged at 4°C for 20 min at 5000g and the supernatant was used for histamine assay.

3.6. Experimental protocol

3.6.1. Study I

After surgery, the animals were allowed to stabilize for 30 min, and the cardiovascular parameters were then recorded to establish the baseline variables (-20 min). In this series of experiments, ET-1 was administered alone or in combination with the ET receptor antagonists. In group 1 (n=6) a solution of 1 nmol/kg ET-1 (Alexis Corp., Läufelfingen, Switzerland) was infused i.v. (0-10 min) into the systemic circulation with a syringe pump, and the hemodynamic parameters were then observed for a further 60 min.

In groups 2-5 (n=6 in each group) the ET-A receptor antagonist BQ-610 (homopiperidynil-carbonyl-Leu-D-Trp(CHO)-D-TRP-OH (Ishikawa 1992), Alexis Corp., 100 nmol/kg (Thompson 1995)), ETR-P1/fl peptide (VLNLCALSVDRYRAVASWRVI, Kurabo Ltd., Osaka, Japan, 100 nmol/kg (Baranyi 1995)), or the ET-B receptor blocker IRL-1038

(Cys11-Cys15-ET-1(11-21), Alexis Corp., 100 nmol/kg (Karaki 1993)) was infused into the systemic circulation over 10 min (-20-10 min). In these groups, 10 min after the end of BQ-610, ETR-P1/fl or IRL-1038 peptide pretreatment, ET-1 in a 1 nmol/kg dose was infused i.v. over 10 min (0-10 min), and the circulatory effects were observed for another 60 min. At the end of the observation period, animals were then killed with an overdose of pentobarbital.

3.6.2. Study II

The animals were randomized to one or other of the following 3 groups. In groups 1 and 2, orthotopic SBTX was performed, as described previously. Group 1 (n=5) served as transplanted control (SBTX group). The animals in group 2 (n=5) were treated with ETR-P1/fl peptide in a dose of 40 nmol/kg intravenously at the onset of reperfusion (SBTX+ETR-P1/fl group). The cold ischemia time was identical in both transplanted groups (60 min). The animals in group 3 (n=5) served as sham-operated controls (Sham-operated group). After a stabilization period, basal cardiovascular parameters were measured for 20 min, then OPS imaging was performed to establish baseline microvascular variables in all groups (-60 min). During the 4 hr reperfusion phase, microcirculatory observations were made in every hour, using OPS imaging in groups 1, 2 and 3. Leukocyte-endothelial cell interactions were also monitored hourly during the 4-hr reperfusion phase by IVM. Due to technical reasons, in these groups no IVM images could be recorded before harvesting.

Animals were infused continuously with Ringer's lactate at a rate of 10 ml/kg/h and received the vehicle for ETR-P1/fl peptide (saline) at matching time points after surgery. At the end of the observation period, the animals were then killed with an overdose of pentobarbital.

3.6.3. Study III

After surgery, the animals were allowed to stabilize for 15 min and the cardiovascular parameters were then recorded to establish the baseline variables (-30 min). The animals were randomly divided into groups. In the first series of experiments, dose responses to ET-1 (Alexis Corporation, Läufelfingen, Switzerland) were obtained. The animals were allotted into the following groups: group 1, sham-operated (n=6); group 2, 0.1 nmol/kg ET-1 (n=5); group 3, 1 nmol/kg ET-1 (n=5); group 4, 3 nmol/kg ET-1 (n=6). In these groups, 30 min after the end of baseline measurements, a solution of 0.1 mL ET-1 or vehicle was infused i.v. into the systemic circulation over 15 min (0-15 min). An additional group of animals (n=5) served

as positive control. In this group, segmental intestinal ischemia was induced by a 15-min occlusion (0-15 min) of the ileal artery.

In the second series, 3 nmol/kg ET-1 was administered in combination with ET receptor blockers. In these groups (n=5 in each one), the ET-A receptor antagonists ETR-P1/fl (300 nmol/kg) and BQ-610 peptides (300 nmol/kg), or the ET-B receptor antagonist IRL-1038 (300 nmol/kg) was infused i.v. for 30 min (-30-0 min), followed by a 15-min of ET-1 infusion (0-15 min) into the systemic circulation after the end of BQ-610, ETR-P1/fl peptide, or IRL-1038 pretreatment, respectively. The circulatory changes were observed for a further 60 min. At the end of the observation period, a tissue sample was taken from the intestinal segment, and the animals were then killed with an overdose of pentobarbital.

3.6.4. Study IV

After surgery, the animals were allowed to stabilize for 15 min, and the cardiovascular parameters were then recorded to establish the baseline variables (-15 min). Group 1 (n=7) served as a saline-treated control. In group 2 (n=7) a solution of 1 nmol/kg ET-1 (Alexis Corp, Läufelfingen, Switzerland) was infused i.v. for 30 min (0-30 min) into the systemic circulation with a syringe pump. In groups 3 (n=7) and 4 (n=7), the ET-A receptor antagonist ETR-P1/fl peptide (100 nmol/kg) or the MC stabilizer Na-cromoglycate (Sigma-Aldrich, 20 mg/kg) were infused i.v. for 10 min (-15-5 min), followed by a 30-min of ET-1 infusion (0-30 min) into the systemic circulation after the end of ETR-P1/fl peptide, or Na-cromoglycate pretreatment, respectively. After infusion, MAP was observed for a further 60 min. At the end of the experiments, tissue biopsies were taken from the ileal part of the small intestine to determine tissue histamine level and MPO enzyme activity and the animals were then killed with an overdose of pentobarbital.

3.7. Statistical analysis

The statistical analysis of our data was performed with non-parametric statistical methods, using a statistical software package (Jandel Scientific, Erkrath, Germany). Statistical differences within the groups, compared to the baseline values, were investigated by using Friedman and Dunn's method. Intergroup comparisons were performed with Kruskal-Wallis and Dunn's method. $p<0.05$ was considered a significant difference. In the Figures and Tables, median values, 25^{th} and 75^{th} percentiles are given. Box plot figures demonstrate the median, the 10^{th} (lower whisker), 25^{th} (bottom of the box), 75^{th} (top of the box) and 90^{th} (upper whisker) percentiles.

4. RESULTS

4.1. Study I - The role of endothelin receptors in the exogenous endothelin-1-induced circulatory changes

The resting hemodynamic parameters were similar in each of the groups studied. The significant differences in the Figures and Table are shown by the following symbols: * $p<0.05$ vs. baseline value (-20 min), # $p<0.05$ vs. ET-1-treated group.

Administration of ET-A receptor antagonist ETR-P1/fl peptide and BQ-610 induced a transient, approximately 10% decrease in MAP and a 10% increase in CO, while the HR values did not change significantly. The ET-B receptor antagonist IRL-1038 did not exert any significant effect on these parameters.

Infusion of 1 nmol/kg ET-1 induced an immediate rise by 250% in TPR (Figure I/1). After 30 min, MAP had increased from 133 to 165 mm Hg (Table I). This hypertension was associated with a significant decrease in CO (Figure I/2).

Figure I/1 Total peripheral resistance

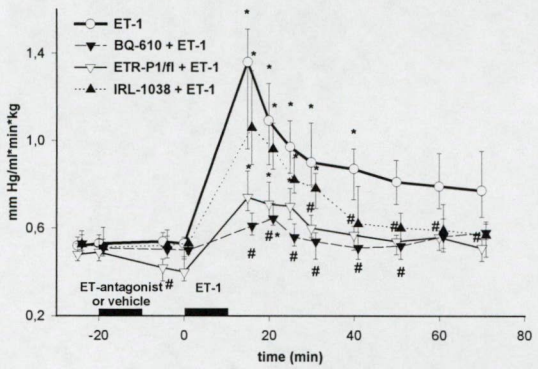
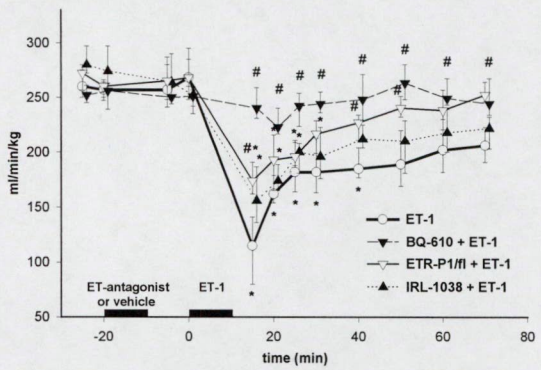


Figure I/2 Cardiac output



Pretreatment with ETR-P1/fl peptide and BQ-610 significantly attenuated the ET-1-induced increase in MAP (Table I) and reduced the decrease in CO. ET-B receptor antagonist IRL-1038 did not influence the ET-1-induced fall in CO (Figure I/2). Similarly, the increase induced in TPR by ET-1 was reduced by BQ-610 and ETR-P1/fl peptide pretreatment, whereas IRL-1038 was ineffective (Figure I/1).

Table I Mean arterial pressure

	Time	ET-1 (n=6)	BQ-610 + ET-1 (n=6)	ETR-P1/fl + ET-1 (n=6)	IRL-1038 + ET-1 (n=6)
	-20 min (Baseline)	133 (127, 140)	126 (122, 134)	124 (104, 132)	134 (130, 136)
MAP	*15 min*	156 (145, 172)	140 (131, 149)	131 (126, 140)	157 (148, 165)
(mm Hg)	*30 min*	165 (156, 178) *	127 (120, 134) #	134 (129, 142) #	152 (144, 161)
	70 min	144 (135, 161)	130 (122, 140)	119 (111, 128)	122 (115, 132)

4.2. Study II - Effects of ET-A receptor blockade on the mesenteric circulatory pattern and leukocyte-endothelial cell interactions following small bowel autotransplantation

The baseline values of the resting hemodynamic parameters were not significantly different among the groups. Comparing to the baseline values, there were no significant hemodynamic changes in the sham-operated group 3, throughout the experimental period.

The statistically different alterations in the Figures and Tables are shown by the following symbols: * $p<0.05$ vs. baseline value (-60 min), x $p<0.05$ vs. sham-operated, # $p<0.05$ vs. SBTX.

However in the SBTX+ETR-P1/fl group SMAP reached lower value at the onset of reperfusion, but this alteration was not significant compared to the time-matching values (Figure II/1). Following transplantation, SMVP increased significantly in the non-treated group (Figure II/2). In this group, SMAF decreased significantly during reperfusion; the calculated MVR values increased progressively (Figures II/3 and 4).

Figure II/1 Segmental arterial pressure

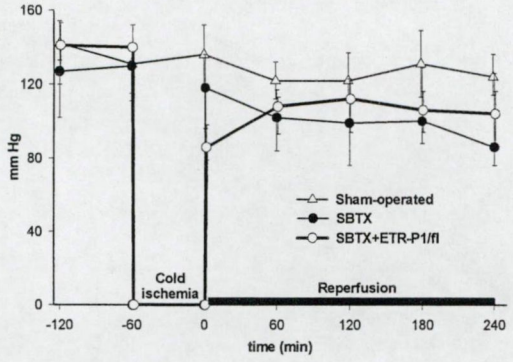
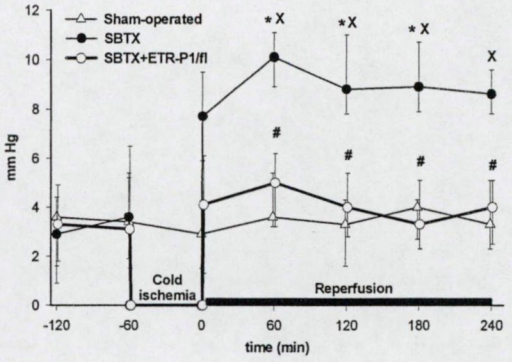


Figure II/2 Segmental venous pressure



In the SBTX+ETR-P1/fl group, the ET-A receptor antagonist treatment significantly inhibited the SBTX-induced mesenteric hemodynamic changes (Figures II/1-4). The infusion of ETR-P1/fl peptide significantly ameliorated the fall in SMAF (Figure II/4). Similarly, the transplantation-induced elevation of MVR was also attenuated after the ETR-P1/fl treatment. (Figure II/3).

Figure II/3 Mesenteric vascular resistance

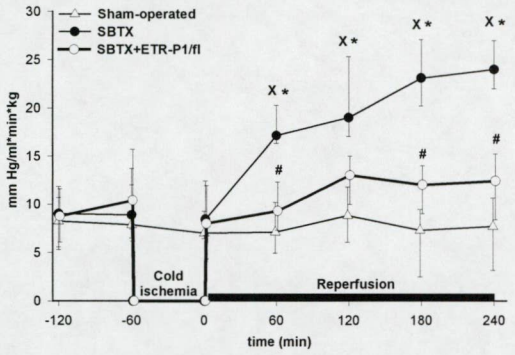
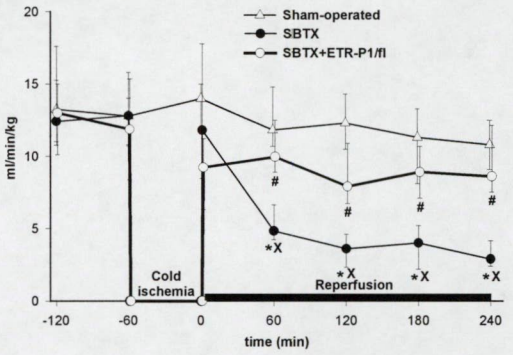


Figure II/4 Mesenteric blood flow



Following transplantation, RBCV decreased slightly, but significantly during the reperfusion phase in both transplanted groups. ETR-P1/fl treatment could not modify this parameter significantly. Neither arteriole, nor venule diameters changed significantly in these groups (Tables II/1 and 2).

Table II/1 Arteriole diameter and red blood cell velocity in the arterioles

	Time	Sham-operated (n=5)	SBTX (n=5)	SBTX+ETR-P1/fl (n=5)
Arteriole diameter (μm)	*-60 min (Baseline)*	10.2 (9.6, 10.6)	10.2 (8.4, 11)	9.8 (8.7, 10.3)
	60 min	10.0 (8.9, 10.3)	9.7 (9.3, 10.1)	10.1 (8.9, 10.7)
	120 min	9.3 (8.8, 10.1)	9.8 (8.9, 11.1)	9.8 (9.2, 10.9)
	180 min	9.7 (9.8, 10.0)	10.3 (10.1, 10.7)	10.3 (9.8, 11.2)
	240 min	10.1 (9.2, 10.6)	10.1 (9.6, 10.9)	9.8 (9.2, 10.4)
RBCV in arterioles (μm/s)	*-60 min (Baseline)*	1035 (999, 1071)	1129 (1020, 1156)	1155 (992, 1218)
	60 min	1178 (1086, 1204)	566 (482, 665) *, x	864 (787, 910) *, x
	120 min	944 (880, 992)	701 (510, 840) *, x	887 (689, 1044) *
	180 min	943 (900, 986)	1119 (860, 1122)	1283 (1007, 1443)
	240 min	916 (831, 1001)	922 (764, 1031) *	743 (636, 850) *

Table II/2 Venule diameter and red blood cell velocity in the venules

	Time	Sham-operated (n=5)	SBTX (n=5)	SBTX+ETR-P1/fl (n=5)
Venule diameter (μm)	*-60 min (Baseline)*	13.1 (10.6, 14.6)	12.1 (10.4, 14.0)	12.8 (11.6, 14.3)
	60 min	11.8 (10.9, 14.3)	11.7 (9.9, 13.1)	12.1 (10.9, 13.7)
	120 min	12.3 (10.8, 13.9)	11.1 (10.9, 14.1)	11.8 (9.9, 14.5)
	180 min	11.7 (9.8, 14.0)	12.3 (10.9, 14.7)	12.1 (11.2, 14.2)
	240 min	12.5 (11.2, 14.2)	11.9 (9.6, 13.9)	11.8 (9.7, 14.4)
RBCV in venules (μm/s)	*-60 min (Baseline)*	559 (419, 700)	678 (608, 745)	688 (625, 709)
	60 min	528 (387, 619)	547 (402, 808)	554 (511, 681)
	120 min	699 (582, 878)	646 (417, 743)	341 (312, 479) *, x
	180 min	800 (617, 890)	494 (399, 620) *, x	407 (261, 589) *, x
	240 min	752 (446, 758)	532 (378, 712) *	416 (333, 500) *

Villus FCD was significantly reduced in the untreated group, compared to the matching time point values. This reduction of FCD was significantly attenuated by the ET-A receptor antagonist treatment (Figure II/5).

In the sham-operated group, epithelial thickness remained unchanged during the observation period. In contrast to it, there was a marked reduction of the epithelial height in both transplanted groups. The ETR-P1/fl treatment attenuated the tapering of the epithelial layer at the 60 and 120 min of reperfusion. In the later timepoints, however the absulute values of epithelial thickness remained higher in the ET-A receptor antagonist-treated than the untreated group, the differences were not statistically different (Figure II/6).

Figure II/5 Functional capillary density

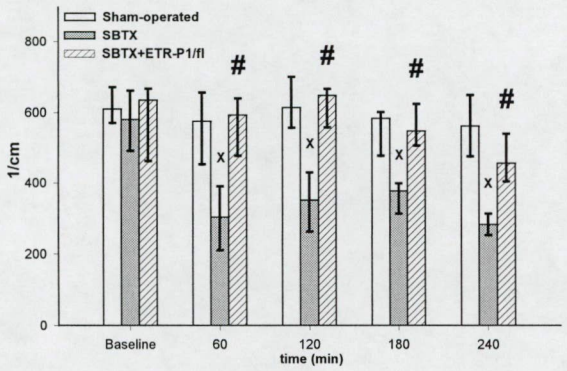
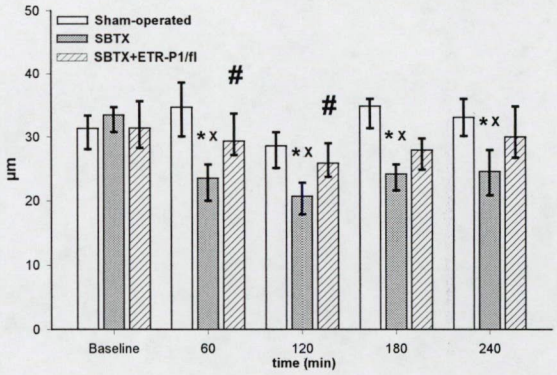


Figure II/6 Epithelial thickness



During reperfusion, a significant leukocyte-endothelial cell interactions were observed in the mesentery. The parameters of leukocyte activation as the percentage of rolling leukocytes and the number of adherent cells in the postcapillary mesenteric venules were significantly elevated, when compared to the values of the sham-operated group. These leukocyte-endothelial cell interactions were reduced after ET-A receptor blockade; both the percentage of rolling cells and the number of adherent cells were significantly less in ETR-P1/fl peptide treated-group (Figures II/7 and 8).

The number of extravasated leukocytes around the postcapillary mesenteric venules was also higher in the transplanted group than at the matching time points in the sham-operated group. The inhibition of the ET-A receptors decreased the number of extravasated leukocytes significantly (Figure II/9).

Figure II/7 Percentage of rolling leukocytes

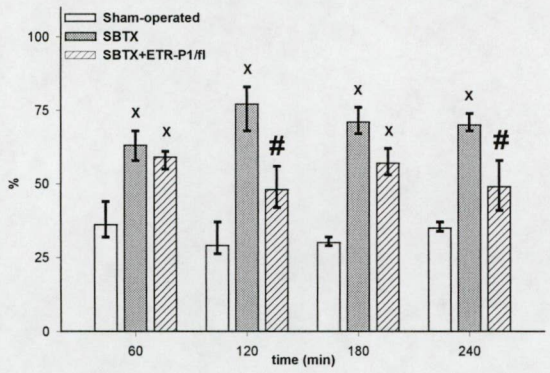


Figure II/8 Number of adherent cells

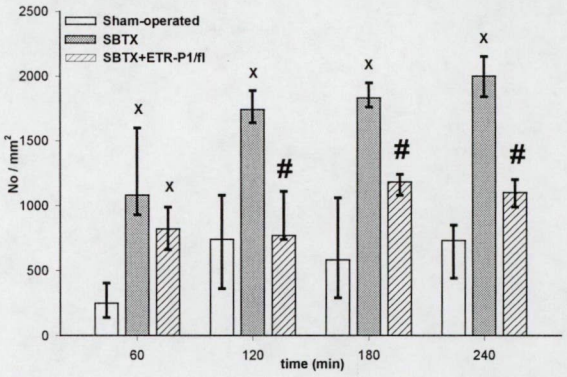
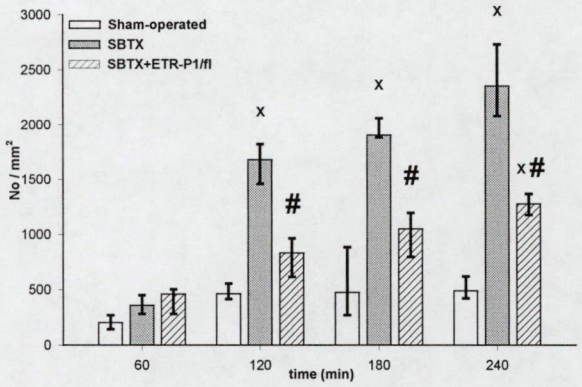


Figure II/9 Number of extravasated leukocytes



4.3. Study III - Mucosal mast cell degranulation in the small intestine

The resting hemodynamic parameters were similar in each of the groups studied. Significant differences in the Figures and in the Tables are shown by the following symbols: * $p<0.05$ vs. baseline value, x $p<0.05$ vs. Sham-operated, # $p<0.05$ vs. 3 nmol/kg ET-1-treated, and § $p<0.05$ vs. IR group.

In the first series of experiments, the increasing doses of ET-1 caused marked changes in intestinal hemodynamics. The intestinal RBC flux displayed a dose-dependent decrease when the MAP was kept elevated. The reduction in intestinal RBC flux was more pronounced in the 3 nmol/kg ET-1-treated animals as compared with those undergoing 0.1 nmol/kg ET-1 treatment (Table III/1).

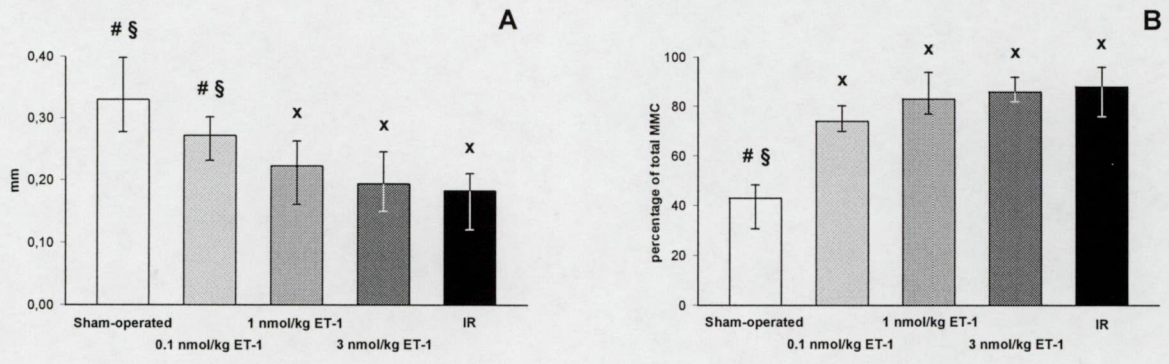
In the sham-operated group, the villus MC count was unchanged and no significant increase in degranulation was observed in biopsies taken at the end of the observation period. A significant, dose-dependent diminution of the villus height was induced by ET-1 infusion as compared to the control group. The shortening of the villi was statistically significant after the administration of 1 or 3 nmol/kg ET-1, and there was a significant difference in this parameter between the 0.1 and 3 nmol/kg ET-1-treated animals (Figure III/1/A). Simultaneously, the

mucosal alterations as assessed on the Chiu scale were statistically different from the control in the 1 and 3 nmol/kg ET-1-treated groups (Figure III/3/A). The MC degranulation ratio exhibited a significant increase after the ET-1 treatment. The ET-1 infusions elevated the proportion of degranulated MCs almost twofold in each of the ET-1-treated groups (Figure III/1/B).

Table III/1 Effects of ET-1 or ischemia-reperfusion (IR) on the mean arterial pressure (MAP) and the small intestinal microcirculation (RBC flux) in the rat

Treatment					
IR	***ET-1 doses*** (nmol/kg i.v.)	***Time***	***n***	***MAP*** (mm Hg)	***RBC flux*** (RBC count x velocity)
-	-	***-30 min (Baseline)***	6	93 (77, 110)	493 (453, 553)
-	-	***15 min***	6	90 (79, 103)	478 (422, 540) #, §
-	-	***75 min***	6	98 (78, 118)	418 (462, 488) #, §
-	**0.1**	***-30 min (Baseline)***	5	100 (96, 112)	431 (389, 518)
-	**0.1**	***15 min***	5	105 (93, 119)	371 (310, 422) #, §
-	**0.1**	***75 min***	5	109 (93, 128)	352 (272, 434) *
-	**1**	***-30 min (Baseline)***	5	94 (79, 114)	523 (442, 562)
-	**1**	***15 min***	5	102 (84, 118)	279 (240, 312) *, x
-	**1**	***75 min***	5	106 (81, 131)	261 (231, 327) *, x
-	**3**	***-30 min (Baseline)***	6	94 (81, 106)	528 (493, 578)
-	**3**	***15 min***	6	110 (93, 131)	212 (184, 244) *, x
-	**3**	***75 min***	6	101 (90, 123)	231 (162 304) *, x
+	-	***-30 min (Baseline)***	4	98 (93, 107)	428 (363, 503)
+	-	***15 min***	4	100 (89, 122)	169 (120, 206) *, x
+	-	***75 min***	4	106 (97, 118)	222 (178, 294) *, x

Figure III/1 ET-1 and ischemia-induced alterations in the villus length (A) and MC degranulation (B)



The ET-A receptor antagonists ETR-P1/fl and BQ-610 peptides attenuated the ET-1-induced fall in RBC flow (Table III/2), the reduction of villus length (Figure III/2/A) and the ET-induced mucosal damage (Figure III/3/B). Similarly, MC degranulation was significantly inhibited by the ET-A receptor antagonist pretreatment (Figure III/2/B).

ET-B receptor antagonist IRL-1038 peptide administration did not influence the 3 nmol/kg ET-1-induced local intestinal hemodynamic and morphological alterations.

Figure III/2 Effect of ET receptor antagonist pretreatment on the villus length (A) and MC degranulation (B) following endothelin-1 infusion

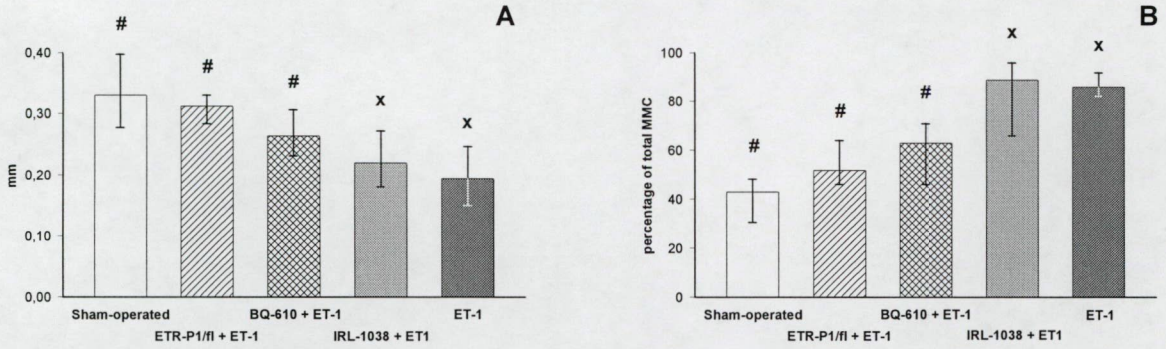


Figure III/3 Grading of mucosal damage according to the standard scale of Chiu

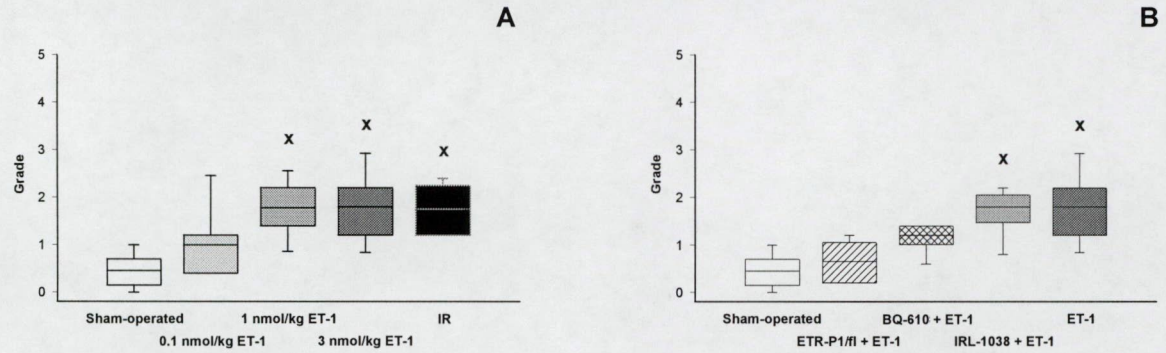


Table III/2 Effects of ET receptor antagonist pretreatment on the ET-1-induced changes in the mean arterial pressure (MAP) and the microcirculation of the rat small intestine (RBC flux)

Treatment					
ET receptor Antagonist	***ET-1*** (3 nmol/kg i.v.)	***Time***	***n***	***MAP*** (mm Hg)	***RBC flux*** (RBC count x velocity)
-	-	***-30 min (Baseline)***	6	93 (77, 110)	493 (453, 553)
-	-	***15 min***	6	90 (79, 103)	478 (422, 540) #
-	-	***75 min***	6	98 (78, 118)	418 (462, 488) #
ETR-P1/fl	+	***-30 min (Baseline)***	5	94 (84, 117)	422 (389, 487)
ETR-P1/fl	+	***15 min***	5	108 (94, 128)	442 (319, 542)
ETR-P1/fl	+	***75 min***	5	93 (74, 122)	418 (283, 503)
BQ-610	+	***-30 min (Baseline)***	5	106 (88, 132)	522 (363, 698)
BQ-610	+	***15 min***	5	115 (98, 142)	452 (342, 561)
BQ-610	+	***75 min***	5	93 (68, 144)	503 (335, 622) #
IRL-1038	+	***-30 min (Baseline)***	6	98 (73, 134)	534 (424, 612)
IRL-1038	+	***15 min***	6	120 (89, 144)	302 (280, 356)
IRL-1038	+	***75 min***	6	98 (71, 122)	318 (244, 412) *
-	+	***-30 min (Baseline)***	6	94 (81, 106)	528 (493, 578)
-	+	***15 min***	6	110 (93, 131)	212 (184, 244) *, x
-	+	***75 min***	6	101 (90, 123)	231 (162 304) *, x

4.4. Study IV - Effects of ET-1 infusion on histamine release and leukocyte activation

Results of this study are under publishing. Baseline MAP values and blood-gas parameters were statistically not different among groups. There was no significant change in these variables of untreated control group throughout the experimental protocol.

Statistical differences in the Table and Figures are shown by the following symbols: * $p<0.05$ within the groups, x $p<0.05$ vs. control group, # $p<0.05$ vs. ET-1-treated group. Infusion of ET-1 induced an approx. twofold rise in TPR, compared to the baseline values. The increase of TPR reached its maximum value at the 30 min and remained elevated significantly by the 60 min. Both the ET-A antagonist ETR-P1/fl peptide and MC-stabilizer Na-cromoglycate administration attenuated the developing TPR-elevation significantly.

Concomitantly with the increase of TPR, CO dropped by approx. 50%. However both applied pretreatment could reach only partial influence on the ET-1-induced CO decrease, they were able to attenuate the fall of CO at the 30 min (Figure IV/1-2).

Figure IV/1 Total peripheral resistance

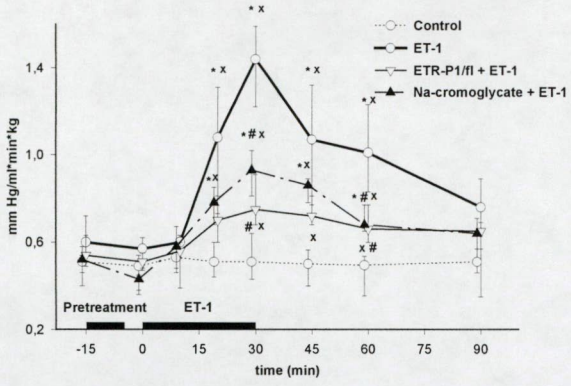
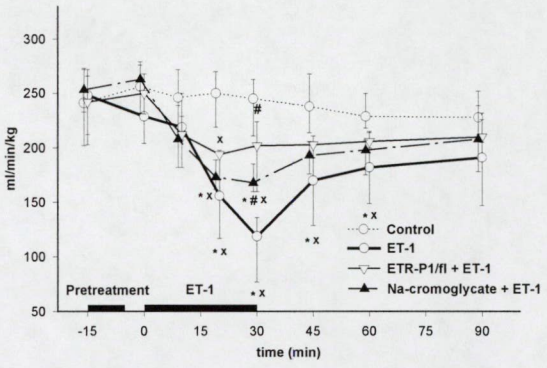


Figure IV/2 Cardiac output



The infusion of 1 nmol/kg ET-1 resulted in a sustained increase in MAP. Neither ETR-P1/fl peptide, nor Na-cromoglycate pretreatment did result statistically different alterations in the ET-1-induced changes in MAP (Table IV).

Table IV Mean arterial pressure

	Time	Control (n=7)	ET-1 (n=7)	ETR-P1/fl + ET-1 (n=7)	Na-cromoglycate + ET-1 (n=7)
	-15 min (Baseline)	128 (114, 134)	131 (117, 151)	118 (116, 119)	134 (112, 135)
	0 min	122 (110, 134)	124 (114, 135)	106 (97, 134)	123 (112, 130)
	10 min	124 (100, 126)	118 (116, 126)	115 (105, 136)	134 (124, 138)
MAP	***20 min***	124 (100, 143)	143 (134, 144) *x	135 (133, 141) *x	145 (133, 156) *x
(mm Hg)	***30 min***	118 (100, 144)	148 (135, 156) *x	142 (127, 152) *x	149 (140, 157) *x
	45 min	104 (93, 120)	144 (136, 155) *x	135 (134, 140) *x	142 (136, 152) *x
	60 min	109 (100, 113)	135 (131, 140)	127 (109, 144)	136 (123, 150)
	90 min	106 (97, 109)	129 (120, 134)	124 (107, 132)	129 (115, 140)

Figures IV/3 and 4 demonstrate the changes in plasma and small bowel histamine levels after 1 nmol/kg ET-1 infusion. Exogenous ET-1 resulted in a twofold elevation of plasma histamine level as compared to the baseline and saline-treated control group values, respectively. Both ETR-P1/fl and Na-cromoglycate pretreatment inhibited the elevation of plasma histamine level significantly (Figure IV/3).

ET-1 treatment induced an approx. 40% decrease in the small bowel histamine level, as compared to the control group. This effect was inhibited by the ET-A receptor antagonist pretreatment. Likewise, following the Na-cromoglycate infusion, a reduced decrease of the tissue histamine level was observed (Figure IV/4).

Figure IV/3 Plasma histamine level

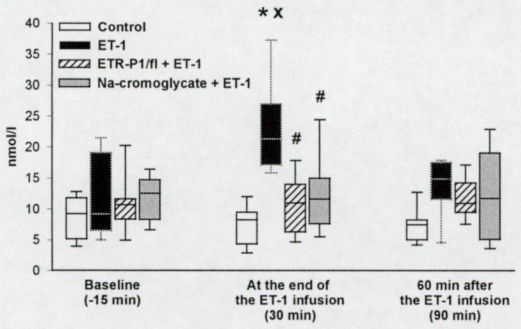
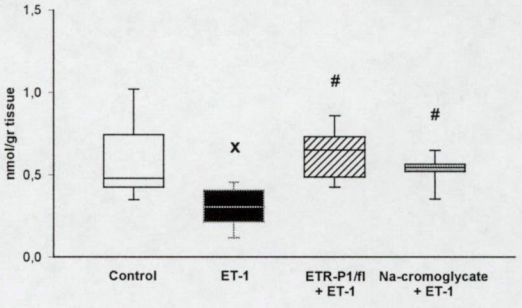
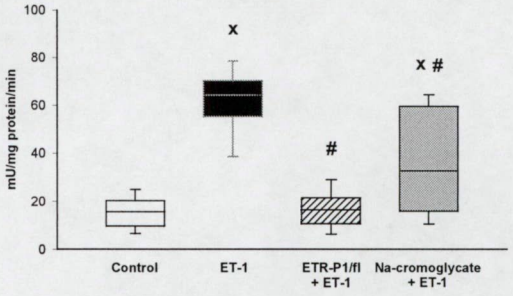


Figure IV/4 Small bowel histamine level



MPO activity was assessed for the indirect quantification of neutrophil accumulation in tissues. ET-1 caused a significant increase in MPO activity in the rat small bowel by the end of the observation period. ETR-P1/fl peptide pretreatment significantly attenuated the ET-1-induced rise of MPO activity in the small bowel. Similarly, the Na-cromoglycate administration was also effective for reducing this ET-1-triggered effect (Figure IV/5).

Figure IV/5 MPO activity in the small bowel



5. DISCUSSION

5.1. Effects of endothelin receptor antagonism on the global hemodynamics

In our experiments we used both ET-A and ET-B receptor antagonists to distinguish the hemodynamic effects mediated by different receptor subtypes. In pilot studies we compared the abilities of ET receptor antagonists to influence the systemic hemodynamics of untreated rats. The ET-B receptor antagonist IRL-1038 alone had no direct effects on any of the systemic parameters measured, but the ET-A receptor antagonist BQ-610 and ETR-P1/fl peptide induced a transient fall in MAP, implicating a role for ET-1 in the maintenance of vascular tone. Thereafter we administered ET-1 to establish a global hemodynamic pattern comparable with the sequence of ischemia-reperfusion-induced circulatory changes (Schlichting 1995, Michida 1994). The 1 nmol/kg ET-1 infusion induced a significant elevation of TPR as a consequence of the systemic vasoconstriction. The reduction of the vascular cross-section resulted in an elevation of MAP, and the CO decreased. This latter effect was due to the insufficient perfusion of the heart. Although *in vitro* data proved the positive inotropic effect of ET, under *in vivo* circumstances the evoked coronary vasoconstriction counteracts this effect.

The attenuated ET-1-evoked systemic hemodynamic effects followed by the ET-A receptor antagonist BQ-610 and ETR-P1/fl peptide suggest a dominantly ET-A receptor mediated hemodynamic effects in the rat circulation. In spite of the statistically not significant differences, we should note that the hemodynamic response to ET-1 infusion was partially inhibited following the IRL-1038 pretreatment. However the predominancy of the ET-A receptors is highly likely, and the partial response to the ET-B-receptor antagonist IRL-1038 suggests a minor role for ET-B_2 receptors in the influence of the ET-1-mediated vasoconstriction.

5.2. Effects of endothelin-A receptor blockade on the transplanted intestinal graft

SBTX is a classical example of an ischemia-reperfusion injury with a significant clinical relevance. Even if the ischemic conditions and reperfusion are controlled in transplantation procedures, the reperfusion injury of the mucosa can contribute to the high complication rate of intestinal transplantation.

Ischemia-reperfusion syndrome is characterized by marked vasoconstriction, free radical-mediated tissue injury and granulocyte accumulation. Since previous data suggested the predominant role of ET-A receptors in the evolving vasoconstriction, in Study II an ET-A antagonist therapy was tested in our model. The experimental setup was designed for the investigation of ischemia-reperfusion-related changes; the autotransplantation enabled the exclusion of immunologic reactions and the separate investigation of the ischemia-reperfusion-related pathologic events following transplantation.

The experimental procedure resulted in significant macrohemodynamic alterations in the small intestine. These changes might be characterized by a marked reduction of SMAF and an increase of MVR. The concomitant rise in SMVP could be a sign of a decreased venous return, thus we presume that postcapillary mesenteric venous congestion also contributes to the repression of the arterial inflow. The marked and sustained increase in SMVP suggests a gradually evolving vasoconstriction in the mesenteric venous system. The development of vasoconstriction at the arterial side is also supposed, even if SMAP remained relatively unchanged. Following ETR-P1/fl peptide administration these alterations were significantly attenuated; the ameliorated SMAF with the attenuated rise of MVR and SMVP reduction refer to the major role of ET-A receptors in the mediation of the evolving vasoconstriction.

It has been demonstrated, that ETR-P1/fl peptide restores the depressed cardiac performance in endotoxemia (Wolfard 2000), thus a positive inotropic effect of the ETR-P1/fl is also conceivable. The experimental instrumentation did not enable to measure this parameter, but if CO was supported by the ET-A receptor antagonist treatment, then it should have manifested in the amelioration of SMAF as well.

The increased macrocirculatory resistance might be explained by the characteristic vasculatory pattern of the villus. Under physiologic conditions, the blood passes the capillaries and returns through the postcapillary venules (Casley-Smith 1984). During reperfusion, the decreased FCD refers a vasoconstriction of the precapillary sphincters; i.e. the capillaries were shunted, and a considerable part of the inflowing blood turned back into the venules directly. Also, the precapillary vasoconstriction can explain the relatively small decrease in villus RBCV in the transplanted group. RBCV is determined primarily by the blood flow and the cross section of the circulatory area. Supporting this hypothesis that no marked difference was found in central arteriolar diameters and red blood cell velocities, therefore the villus blood flow is not primarily influenced by the vasoconstriction of the central villus arteriole. Thus the net reduction of the supplying blood flow might be not the

primary reason but a consequence of the perfusion insufficiency: the closure of the capillaries and the evolving shunt circulation resulted in an enhanced reduction in the cumulative cross section of the mesenteric circulatory area.

The microcirculatory impairment, reflecting in the reduction of FCD might be also the result of the no-reflow phenomenon. This multifactorial event is facilitated by interstitial edema, endothelial swelling and leukocyte plugging of the microvasculature (Menger 1997). There is a growing body of evidence that ETs can facilitate this microcirculatory reaction. Through the activation of ET-A receptors, ET-1 can increase vascular permeability and induce interstitial edema via the activation of ET-A receptors (Filep 1992). Likewise, the supposed role of ETs in the no-reflow phenomenon is also supported by data reporting ET-1-induced leukocyte-endothelial cell interactions in submucosal venules of the rat (Boros 1998). Our current results also provide evidence for the proadhesive effect of endogenously liberated ET-1; reperfusion was accompanied by increased leukocyte rolling and adherence in the mesenteric postcapillary venules and this change was significantly attenuated by the ET-A antagonist therapy.

Similarly, during reperfusion a pronounced granulocyte deposition was observed in the mesentery as well. This granulocyte extravasation could be also attenuated significantly by the ETR-P1/fl peptide treatment. This coincides with previous observation (Boros 1998), of ET-1-induced leukocyte-endothelial interactions via the ET-A receptors. According to it, MPO activity, a marker of tissue granulocyte deposition also indicated an ET-A receptor dependent rise in the transplanted intestinal segment (Wolfard 1999). These data, together with our results, suggest that endogenously produced ETs enhanced leukocyte-endothelial interactions and subsequent tissue granulocyte accumulation in our model significantly.

Beyond the hemodynamic alterations and inflammatory cell deposition, consequences of the transplantation are manifested in morphological alterations as well. In contrast to previous histological observations, the OPS imaging technique revealed an instant closure of the injured epithelial layer, therefore in this way we did not observe exposed "denuded" or "amputated" villi, which were characteristic in light microscopy. The thickening of the epithelial layer reveals an incomplete regeneration during the reperfusion phase. The effectiveness of ET-A receptor antagonism in the protection against mucosal damage demonstrates the importance of the ETs in the induction of mucosal lesions. The ETR-P1/fl peptide improved tissue perfusion by decreasing MVR, probably via the reduction of vasoconstriction, and might therefore be able to attenuate the hypoperfusion-related tissue injury. On the other hand, the treatment decreased the intramucosal deposition of

granulocytes. Since the extravasated inflammatory cells may contribute significantly to tissue injury, through the liberation of cytolytic enzymes and free radical production, the decreased leukocyte accumulation could also contribute to the protective effect of ETR-P1/fl peptide.

In Study II we tried to prove the effectiveness of an ET-A receptor antagonist therapy on the development of the ischemia-reperfusion injury. For this purpose we designed a model both focusing an ischemia-reperfusion-related challenge and mimicking a potential therapeutic strategy; however we must note that the relatively short follow-up period and the exclusion of the immunologic consequences weaken our conclusions. In the face of the limitations we can state that ETs play a significant role in the mediation of ischemia-reperfusion injuries following small bowel transplantation. The applied ET-A receptor antagonist therapy reduced vasoconstriction, inhibited leukocyte-endothelial cell interactions, thus improved tissue perfusion and attenuated epithelial damage of the villi.

5.3. Role of mast cells in the endothelin-induced tissue reactions in the rat small intestine

Low-flow conditions are accompanied by the early release of proinflammatory compounds. MCs might be a source of these mediators, which may be involved in the secondary vascular reactions such as leukocyte recruitment, vasodilation and permeability changes (Galli 1993, Gaboury 1995, Szabo 1997). There is a growing body of evidence that MCs may contribute to reperfusion injury and leukocyte recruitment in the reperfused intestine (Kanwar 1994, Kurose 1994). Following mesenteric ischemia, the release of histamine from intestine also suggests an increased MC degranulation (Boros 1991). Additionally, during reperfusion, MC degranulation has been shown both histologically (Kurose 1994) and biochemically (Kanwar 1994) in the intestinal tract of the rat. There is a growing body of evidence of a direct ET-triggered histamine release from MCs *in vitro* (Uchida 1992, Yamamura 1994, Egger 1995). Based on these *in vivo* and *in vitro* results, in Studies III and IV we tried to support the concept that MCs play a significant role in the pathogenesis of the ET-induced tissue reactions under *in vivo* circumstances as well.

In our experiments, the applied doses of ET-1 showed comparable alterations to an ischemia-reperfusion challenge. Separating the ET receptor subtype-mediated effects with different ET receptor antagonist application, our data suggest the predominance of ET-A receptors in MC degranulation, neutrophil activation and subsequent tissue damage.

ETs can evoke MC degranulation in different pathways. One possibility might be a direct effect of ET on MCs. This hypothesis is supported by the results of Liu, demonstrating ET-A expression on the surface of MCs in the rat (Liu 1998). An another explanation could be a direct interaction between MC degranulating peptide (MDP) and G-proteins in MCs (Mousli 1991). Considering the very close structural similarities between ET-1 and MDP (Perkins 1990), a similar connection between ET-1 and G-proteins in MCs might be suggested.

The altered osmolarity of the intestinal mucosa as a result of localized perivascular edema might also be a possible mechanism for the ET-induced MC degranulation. The fragility of the MC membranes to osmotic stress has been demonstrated. Intraperitoneal injection of hypotonic salt solutions results in MC membrane destruction, without induction of the osmotic disruption of other cell types (Fawcett 1955). Accordingly, Filep et al. have shown that ET-1 causes dose-dependent increases in vascular permeability through the activation of ET-A receptors as a consequence of the disruption of the endothelial barrier (Filep 1992). Following the decrease in arterial inflow, the declining energy supply for active membrane transport processes and the lack of removal of metabolites may be accompanied by a rapid fluid movement from the vascular lumen to the lamina propria. An acute circulatory breakdown may therefore rapidly cause perivascular edema, leading to MC degranulation.

The imbalance between ET and nitric-oxide can also lead MC activation. This hypothesis is supported by observations reporting the inhibition of nitric-oxide release as a stimulating effect for MC degranulation (Kanwar 1994, Kurose 1994). Similarly, the profound ET-A receptor-mediated vasoconstriction and the ensuing ischemic injury can result in the same effect.

The structural injury of the small intestinal mucosa may be directly connected with the hemodynamic consequences of ET administration. The mucosal lesions were similar to those described in animal models of intestinal ischemia-reperfusion or after comparable doses of exogenous ET-1 (Massberg 1998). In this case, tissue hypoxia, or oxygen-derived free radicals generated during local ischemia-reperfusion injuries could also be MC-degranulating factors.

Increased ET-1 formation and neutrophil accumulation are coexisting phenomena in pathological circumstances such as ischemia-reperfusion injury (Espinosa 1996). It has been also shown that ET-1 stimulates neutrophil adhesion to cultured endothelial cells by the effect on the expression of CD18 and CD11b on the neutrophil surface and ICAM-1 on the endothelial cells (Lopez Farre 1993, Hayasaki 1996). The ET-A receptor triggered neutrophil

migration has been also reported (Elferink 1994). Under *in vivo* circumstances, Boros demonstrated the ET-1-induced rolling and adherence via predominantly ET-A receptors (Boros 1998). Oktar et al. provided an other *in vivo* evidence of the role of leukocytes in the ET-1 intestinal mucosal injury (Oktar 2000). These data are consonant with our results, revealing ET-A receptor leukocyte accumulation and tissue damage, respectively.

Although the vascular endothelium is the most likely source of the generation of ET, we must note that that MCs, monocytes and macrophages can also generate ET (Ehrenreich 1992, Liu 1998). In addition, a release of MC-derived proteases can also influence ET formation via the stimulation of the ET converting enzyme. It has been proven that chymase from lung MCs is involved in the extracellular procession of from big-ET to ET-1, thus contributeing to the elevated ET level following pathological stimuli (Wypij 1992). Similarly, MC-derived histamine can also induce ET production with a positive feedback mechanism in the lung (Ackerman 1995).

MCs can also serve as an ignition key of neutrophil activation (Kanwar 1994). Among other MC-derived cytokines, histamine is a feasible candidate for a mediating agent leading to neutrophil activation. These hypotheses are confirmed by data showing exogenous histamine-triggered neutrophil-endothelial cell interactions. Some of these publications lead to the conclusion that the induction of P-selectin expression is the key event of this histamine-mediated process (Jones 1993, Kubes 1994, Ley 1994). However data are inconsistent about the role of histamine receptor subtypes responsible for this action. While the data of Kubes et al. indicate that neutrophil activation is an H1-receptor mediated effect, Ley et al. demonstrated the involvement of H2 receptors in triggering leukocyte-endothelial cell interactions (Kubes 1994, Ley 1994). Naturally, due to the numerous mediators stored and released by MCs, there are several other possible pathways for neutrophil activation via MC degranulation. Without the assignment of all potential mediators we just mention leukotrienes, PAF, and TNF-α as possible MC-released cytokines involved in neutrophil activation (Lorant 1991, Goldman 1992, Galli 1993, Gaboury 1995).

Administration of the MC membrane stabilizer Na-cromoglycate inhibited not only the ET-1-induced histamine liberation, but influenced the systemic circulatory response of ET-1 as well. This observation suggests that perivascular MCs take a part in the development of the net circulatory response evoked by ET-1. According to our current knowledge, Na-cromoglycate inhibits both Ca^{++} and Cl^- transport across the MC membrane thus preventing MC degranulation (Foreman 1976, Spataro 1976). Based on its particular anionic properties, Na-cromoglycate is also able to inhibit G-protein activation (Klinker 1997). Since ET

receptors are the members of the G-protein receptor superfamily and they take their effects via both Ca^{++} and Cl^- channels (Rubanyi 1994), the Na-cromoglycate-mediated blockade of Cl^- channels, and the subsequent inhibition of voltage-dependent Ca^{++} channels could be a possible explanation for the attenuated hemodynamic response to ET-1 following Na-cromoglycate administration (Takenaka 1992). Although these pathways are rather unspecific, we can not exclude the possibility that the effects of Na-cromoglycate are mediated not exclusively by MC membrane stabilization, but via an ET-receptor-linked mechanism as well.

In conclusion, our data demonstrate that significant endogenous ET-induced leukocyte activation occurs after small bowel transplantation. Our results provide additional evidence of a direct effect of ET on leukocyte-endothelial cell interactions, and prove the contribution of MCs to neutrophil activation in this organ. The supposed common target of Na-cromoglycate and ET on the ET-mediated hemodynamic effects suggests a possible therapeutic potential of Na-cromoglycate in pathophysiological states accompanied by an increased ET production. Although we could examine only a fragment of the possible cellular interactions, the ET release – MC degranulation – leukocyte activation pathway seems to have a decisive role in the reoxygenation injuries of the intestinal tract.

6. ACKNOWLEDGMENTS

I am grateful to Professors Sándor Nagy and Mihály Boros for providing me with the opportunity to work in the Institute of Surgical Research and for their valuable scientific guidance and help.

I am indebted to Dr. József Kaszaki who helped me greatly to learn the necessary surgical and experimental skills. I also thank to all of my colleagues for their contributions to my experimental work.

Special thanks are due to all the technical staff of the Institute. Their skillful help and enthusiastic work have helped me overcome many difficulties and have facilitated the experiments.

This work was supported by research grants OTKA 16889, OTKA T023089, HHMI 75195-54150, MKM 0093/1997, AMFK III. 144/94, and ETT 606/96T-07.

7. REFERENCES

Ackerman V, Carpi S, Bellini A, Vassalli G, Marini M, Mattoli S: Constitutive expression of ET in bronchial epithelial cells of patients with symptomatic and asymptomatic asthma and modulation by histamine and interleukin-1. *J Allergy Clin Immunol* 96(5Pt1):618-627, 1995.

Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S: Cloning and expression of cDNA encoding an endothelin receptor. *Nature* 348:730-732, 1990.

Asako H, Kurose I, Wolf R, DeFrees S, Zheng ZL, Philips ML, Paulson JC, Granger DN: Role of H1 receptors and P-selectin in histamine-induced leukocyte rolling and adhesion in postcapillary venules. *J Clin Invest* 93(4):1508-1515, 1994.

Bahrami S, Yao YM, Leichtfried G, Redl H, Schlag G, Di Padova FE: Monoclonal antibody to endotoxin attenuates hemorrhage-induced lung injury and mortality in rats. *Crit Care Med* 25(6):1030-1036, 1997.

Baranyi L, Campbell W, Oshima K, Fujimoto S, Boros M, Okada H. The antisense homology box: A new motif within proteins that encodes biologically active peptides. *Nat Med* 1:894-901, 1995.

Beaven MA, Horakova Z: The enzymatic isotopic assay of histamine. In Rocha e Silva M (ed*): "Handbook of Experimental Pharmacology."* Berlin: Springer-Verlag, 18:151, 1978.

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

Boros M, Kaszaki J, Nagy S: Oxigen free-radical induced histamine release during intestinal ischemia and reperfusion. *Eur Surg Res* 21(6):297-304, 1989.

Boros M, Massberg S, Baranyi L, Okada H, Messmer K: Endothelin-1 induces leukocyte adhesion in submucosal venules of the rat small intestine. *Gastroenterology* 114:103-114, 1998.

Boros M, Takaichi S, Hatanaka K: Ischemic time-dependent changes and reperfusion injury in the rat small intestine. *J Surg Res* 59:311-320, 1995.

Boros M, Takaichi S, Masuda J, Newlands GF, Hatanaka K: Response of mucosal mast cells to intestinal ischemia-reperfusion injury in the rat. *Shock* 3(2):125-131, 1995.

Browne BJ, Johnson CP, Edmiston CE, Hlava MA, Moore GH, Roza AM, Telford GL, Adams MB: Small bowel transplantation promotes bacterial overgrowth and translocation. *J Surg Res* 51:512-517, 1991.

Burns AR, Bowden RA, Abe Y, Walker DC, Simon SI, Entman ML, Smith CW.: P-selectin mediates neutrophil adhesion to endothelial cell borders. *J Leukoc Biol* 65(3):299-306, 1999.

Casley-Smith JR., Gannon BJ.: Intestinal microcirculation: spatial organization and fine structure. In: "*Physiology of the intestinal circulation.*" Raven Press 305-319, 1984.

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* 101:478-483, 1970.

Clozel M, Clozel JP: Effects of endothelin on regional blood flow in squirrel monkeys. *J Pharmacol Exp Ther* 250:1125-1131, 1989.

Clozel M, Gray GA, Breu V, Löfflet BM, Osterwalder R: The endothelin ET-B receptor mediates both vasodilation and vasoconstriction in vivo. *Biochem Biophys Res Commun* 186:867-873, 1992.

Davenport AP, O'Reilly G, Kuc RE: Endothelin ET-A and ET-B mRNA and receptors expressed by smooth muscle in the human vasculature: majority of the ET-A sub-type. *Br J Pharmacol* 114:1110-1116, 1995.

Egger D, Geuenich S, Denzlinger C, Schmitt E, Mailhammer R, Ehrenreich H, Dormer P, Hultner L: IL-4 renders mast cells functionally responsive to endothelin-1. *J Immunol* 154(4):1830-1837, 1995.

Ehrenreich H, Burd PR, Rottem M, Hultner R, Hylton JB, Garfield M, Coligan JE, Metcalfe DD, Fauci AS: Endothelins belong to the assortment of mast cell-derived and mast cell-bound cytokines. *New Biol* 4:147-156, 1992.

Elferink JG, de Koster BM: Endothelin-induced activation of neutrophil migration. *Biochem Pharmacol* 48(5):865-871, 1994.

Enerbäck L: The mast cell system. in "*Heparin*" (eds: Lane DA, Lindahl U.) Edward Arnold, 97-113, 1989.

Eppihimer MJ, Granger DN: Ischaemia/reperfusion-induced leukocyte-endothelial interactions in postcapillary venules. *Shock* 8:16-25, 1997.

Espinosa G, Lopez Farre A, Cernadas MR, Manzarbeitia F, Tan D, Digiuni E, Mosquera JR, Monton M, Millas I, Hernando L, Casado S, Caramelo C: Role of endothelin in the pathophysiology of renal ischemia-reperfusion in normal rabbits. *Kidney Int* 50(3):776-782, 1996.

Fawcett DW: An experimental study of mast cell degranulation and regeneration. *Anat Rec* 121:29-51, 195.

Ferri C, Bellini C, DeAngelis C, DeSiatri L, Perrone A, Properzi G, Santucci A: Circulating endothelin-1 concentrations in patients with chronic hypoxia. *J Clin Pahol* 48(6):519-524, 1995.

Filep JG, Foldes-Filep E, Rousseau A, Fournier A, Sirois P, Yano M: Endothelin-1 enhances vascular permeability in the rat heart through the ETA receptor. *Eur J Pharmacol* 219(2):343-344, 1992.

Filep JG, Földes-Filep E, Rousseau A, Fournier A, Sirois P, Yano M: Endothelin-1 enhances vascular permeability in the rat heart through the ET_A receptor. *Eur J Pharmacol* 219:343-344, 1992.

Foreman JC, Garland LG: Cromoglycate and other antiallergic drugs: a possible mechanism of action. *Br Med J* 1(6013):820-821, 1976.

Frangogiannis, N.G., Lindsey M.L., Michael L.H., Youker K.A., Bressler R.B., Mendoza L.H., Spengler R.N., Smith C.W., Entman M.L.: Resident cardiac mast cells degranulate and release preformed TNF-α initiating the cytokine cascade in experimental canine myocardial ischemia/reperfusion. *Circulation* 98:699-710, 1998.

Frezza EE, Tzakis A, Fung JJ, Van Thiel DH: Small bowel transplantation: Current progress and clinical application. *Hepato-Gastroenterology* 43:363-376, 1996.

Furchott RF, Zawadski JV: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288:373-376, 1980.

Gaboury JP, Johnston B, Niu XF, Kubes P:: Mechanism underlying acute mast cell-induced leukocyte rolling and adhesion in vivo. *J Immunol* 154:804-813, 1995.

Galli S.J.: New concepts about the mast cell. *N. Engl. Med. J.* 328:257-265, 1993.

Galli SJ, Gordon JR, Wershil BK: Mast cell cytokines in allergy and inflammation. *Agents Actions Supplement* 43:209-20, 1993.

Gardiner SM, Kemp PA, March JE, Bennett T.: Effects of bosentan (Ro 47-0203), an ETA-, ETB-receptor antagonist, on regional haemodynamic responses to endothelins in conscious rats. *Br J Pharmacol* 112(3):823-830, 1994.

Goldman G, Welbourn R, Klausner JM, Kobzik L, Valeri CR, Shepro D, Hechtman HB: Mast cells and leukotrienes mediate neutrophil sequestration and lung edema after remote ischemia in rodents. *Surgery* 112(3):578-586, 1992.

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* 58:403-408, 1994.

Granger DN: Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. *Am J Physiol* 255:H1269-H1275, 1988.

Grant D: Current results of intestinal transplantation. *Lancet* 347:1801-1803, 1996.

Guarnieri C, Melandri G, Caldarera I, Schaeda M, Ligabue A, Guizzardi S, Branzi A: Reduced oxidative activity of circulating neutrophils in patients after myocardial infarction. *Cell Biochem Funct* 8:157-162, 1990.

Hayasaki Y, Nakajima M, Kitano Y, Iwasaki T, Shimamura T, Iwaki K: ICAM-1 expression on cardiac myocytes and aortic endothelial cells via their specific endothelin receptor subtype. *Biochem Biophys Res Commun* 229(3):817-824, 1996.

Hill, JS, Ganellin CR, Timmerman H, Schwartz JC, Shankley NP, Young JM, Schunack W, Levi R, Haas HL: International Union of Pharmacology. XIII. Classification of histamine receptors. *Pharmacol Rev* 49(3):253-278, 1997.

Ihara M, Ishikawa K, Fukuroda T, Saeki T, Funabashi K, Fukami T, Suda H, Yano M. : In vitro biological profile of a highly potent novel endothelin (ET) antagonist BQ-123 selective for the ETA receptor. *J Cardiovasc Pharmacol* 20 Suppl 12:S11-14, 1992.

Irani A.M., Schwartz L.B.: Human mast cell heterogenity. *Allergy Proc.* 15:303-308, 1994.

Ishida K, Takeshige K, Minakami S: Endothelin-1 enhances superoxide generation of human neutrophils stimulated by the chemotactic peptide N-formyl-methionyl-phenylalanine. *Biochem Biophys Res Commun* 173:496-500, 1990.

Ishikawa K, Fukami T, Nagase T: Endothelin antagonistic peptide derivatives with high selectivity for ET-A receptors. *Peptides* 685-686, 1992.

Jones DA, Abbassi O, McIntire LV, McEver RP, Smith CW: P-selectin mediates neutrophil rolling on histamine-stimulated endothelial cells. *Biophys J* 65(4):1560-1569, 1993.

Kanwar S, Hickey MJ, Kubes P: Postischemic inflammation: a role for mast cells in intestine but not in skeletal muscle. *Am J Physiol* 275(2Pt1):G212-218, 1998.

Kanwar S, Johnston B, Kubes P: Leukotriene C4/D4 induces P-selectin and sialyl Lewis(x)-dependent alterations in leukocyte kinetics in vivo. *Circ Res* 77(5):879-887, 1995.

Kanwar S, Kubes P: Ischemia/reperfusion-induced granulocyte influx is a multistep process mediated by mast cells. *Microcirculation* 1(3):175-182, 1994.

Kanwar S, Kubes P: Mast cells contribute to ischemia-reperfusion-induced granulocyte infiltration and intestinal dysfunction. *Am J Physiol* 1994 267(2Pt1):G316-321, 1994.

Kanwar S, Wallace JL, Befus D, Kubes P: Nitric oxide synthesis inhibition increases epithelial permeability via mast cells: *Am J Physiol* 266(2Pt1):G222-229, 1994.

Karaki H, Sudjarwo SA, Hori M, Sakata K, Urade Y, Takai M, Okada T: ETB receptor antagonist, IRL 1038, selectively inhibits the endothelin-induced endothelium-dependent vascular relaxation. *Eur J Pharmacol* 231(3):371-374, 1993.

Kitamura Y, Kasugai T, Nomura S, Matsuda H.: Development of mast cells and basophils. In:" *Foreman J.C., ed: Immunopharmacology of mast cells and basophils.*" Academic Press, London, 5-27, 1993.

Klinker JF, Seifert R: Morphine and muscle relaxants are receptor-independent G-protein activators and cromolyn is an inhibitor of stimulated G-protein activity. *Inflamm Res* 46(2):46-50, 1997.

Kocoshis SA: Small bowel transplantation in infants and children. *Gastroenterol Clin North Am* 23:727-242, 1994.

Kubes P, Kanwar S: Histamine induces leukocyte rolling in post-capillary venules. A P-selectin-mediated event. *J Immunol* 152(7):3570-3577, 1994.

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* 16:89-97, 1996.

Kurose I, Wolf R, Grisham MB, Granger DN: Modulation of ischemia/reperfusion-induced microvascular dysfunction by nitric oxide. *Circ Res* 74(3):376-382, 1994.

Ley K: Histamine can induce leukocyte rolling in rat mesenteric venules. *Am J Physiol* 267(3Pt2):H1017-1023, 1994.

Li X, Steeber DA, Tang ML, Farrar MA, Perlmutter RM, Tedder TF.: Regulation of L-selectin-mediated rolling through receptor dimerization. *J Exp Med* 188(7):1385-1390, 1998.

Liu Y, Yamada H, Ochi J: Immunocytochemical studies on endothelin in mast cells and macrophages in the rat gastrointestinal tract. *Histochem Cell Biol* 109(4):301-307, 1998.

Lock G, Scholmerich J: Non-occlusive mesenteric ischemia. *Hepatogastroenterology* 42:234-239, 1995.

Lojek A, Ciz M., Slavikova H, Duskova M, Vondracek J, Kubala L, Racz I, Hamar J: Leukocyte mobilization, chemiluminescence response, and antioxidative capacity of the blood in intestinal ischemia and reperfusion. *Free Radic Res* 27:359-367, 1997.

Lopez Farre A, Rieso A, Espinosa G, Digiuni E, Cernadas MR, Alvarez V, Monton M, Rivas F, Gallego MJ, Egido J: Effect of endothelin-1 on neutrophil adhesion to endothelial cells and pervused heart. *Circulation* 88(3):1166-1171, 1993.

Lorant DE, Patel KD, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA: Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. *J Cell Biol* 115(1):223-234, 1991.

Lorenz W, Schauer A, Heitand S, Calvoer R, Werle E: Biochemical and histochemical studies on the distribution of histamine in the digestive tract of man, dog and other mammals. *Naunyn-Schmiedebergs Arch Pharmacol* 265:81-100, 1969.

Maclean MR, Randall MD, Hiley CR: Effects of moderate hypoxia, hypercapnia and acidosis on haemodynamic changes induce endothelin-1 in the pithed rat. *Br J Pharmacol* 98:105-1065, 1989.

Masini E, Bianchi S, Gambassi F, Palmerani B, Pistelli A, Carlomagno L, Mannaioni PF: Ischemia reperfusion injury and histamine release in isolated and perfused guinea-pig heart: pharmacological interventions. *Agents Actions* 30(1-2):198-201, 1990.

Massberg S, Boros M, Leiderer R, Baranyi L, Okada H, Messmer K: Endothelin (ET)-1 induced damage in the rat small intestine: role of ET_A receptors. *Shock* 9:177-183, 1998.

Menger MD, Ruecker M, Vollmar B: Capillary dysfunction in striated muscle ischemia/reperfusion: on the mechanisms of capillary "no-reflow". *Shock* 8:2-7, 1997.

Michida T, Kawano S, Masuda E, Kobayashi I, Nishimura Y, Tsujii M, Hayashi N, Takei Y, Tsuji S, Nagano K: Role of endothelin 1 in hemorrhagic shock-induced gastric mucosal injury in rats. *Gastroenterology* 106(4):988-993, 1994.

Minkes RK, Kadowitz PJ: Influence of endothelin on systemic arterial pressure and regional blood flow in the cat. *Eur J Physiol* 163:163-166, 1989.

Mitsuoka H, Unno N, Sakurai T, Kaneko H, Suzuki S, Konno H, Terakawa S, Nakamura S. : Pathophysiological role of endothelins in pulmonary microcirculatory disorders due to intestinal ischemia and reperfusion. *J Surg Res* 87(2):143-151, 1999.

Moncada S, Palmer RMJ, Higgs EA: Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43:109-142, 1991.

Moncada S, Radomski MW, Palmer RMJ: Endothelium-derived relaxing factor: identification as nitric oxideand role in the control of vascular tone and platelet function. *Biochem Pharmacol* 37:2495-2501, 1988.

Moreland S, McMullen D, Abboa-Offei B, Seymour A: evidence for differential location of vasoconstrictor endothelin receptors in the vasculature. *Br J Pharmacol* 12:704-708, 1994.

Mousli M, Bronner C, Bueb JL, Landry Y: Evidence for the interaction of mast cell-degranulating peptide with pertussis toxin-sensitive G proteins in mast cells. *Eur J Pharmacol* 207(3):249-255, 1991.

Mousli M, Bueb JL, Rouot B, Landry Y, Bronner C: G-proteins as targets for non-immunological histamine releasers. *Agents Actions* 33(1-2):81-83, 1991.

Oktar BK, Coskun T, Bozkurt A, Yegen BC, Yuksel M, Haklar G, Bilsel S, Aksungar FB, Cetinel U, Granger DN, Kurtel H: Endothelin-1-induced PMN infiltration and mucosal dysfunction in the rat small intestine. *Am J Physiol* 279(3):G483-491, 2000.

Oktar BK, Gulpinar MA, Bozkurt A, Ghandour S, Cetinel S, Moini H, Yegen BC, Bilsel S, Granger DN, Kurtel H.: Endothelin receptor blockers reduce I/R-induced intestinal mucosal injury: role of blood flow. *Am J Physiol* 282(4):G647-655, 2002.

Parks DA, Granger DN: Contributions of ischemia and reperfusion to mucosal lesion formation. *Am J Physiol* 250:G749-G753, 1986.

Perkins TD, Hider RC, Barlow DJ: Proposed solution structure of endothelin. *Int J Pept Prot Res* 36(2):128-133, 1990.

Pirenne J: Intestinal transplantation: a clinical reality in 1998. *Bull Mem Acad R Med Belg* 153:171-178, 1998.

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 Pediatric Surg* 33:243-254, 1998.

Rosen LA, Hollis TM, Sharma MG: Alterations in bovine endothelial histidine decarboxylase activity following exposure to shearing stresses. *Exp Mol Pathol* 20:329-343, 1974.

Rossi G, Gatti S, Reggiani P, Orsenigo R, Maggi U, Franco P, Marelli O, Perego C, Colledan M, Gridelli B, Quarenghi E, Di Mauro P, Doglia M, Fassati LR, Galmarini D: Results of orthotopic liver-small bowel transplantation in the pig using different immunosuppressive regimens. *Transplant Proc* 28:2519-2522, 1996.

Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacological Reviews* 46(3):325-415, 1994.

Ryan MJ, Brody MJ: Neurogenic and vascular stores of histamine in the dog. *J Pharmacol Exp Therap* 181:83-91, 1972.

Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, Masaki T: Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* 348:732-735, 1990.

Schlichting E, Aspelin T, Grotmol T, Lyberg T: Endothelin and hemodynamic responses to superior mesenteric artery occlusion shock and hemorrhagic shock in pigs. *Shock* 3:109-115, 1995.

Schoenberg MH, Poch B, Younes M, Schwarz A, Baczako K, Lundberg C, Haglund U, Beger HG: Involvement of neutrophils in postischemic damage of the small intestine. *Gut* 32:905-912, 1991.

Shetty SS, Okada T, Webb RL, DelGrande D, Lappe RW: Functionally distinct endothelin-B receptors in vascular endothelium and smooth muscle. *Biochem Biophys Res Commun* 191:459-467, 1993.

Spada M, Alessiani M, Fabbi M, Magnino S, Gelmetti D, Vaccarisi S, Rademacher J, Costa A, Dionigi P, Zonta A: Bacterial translocation is enhanced in pig intestinal transplantation when the colon is included in the graft. *Transplant Proc* 28:2658-2659, 1996.

Spataro AC, Bosmann HB: Mechanism of action of disodium cromoglycate--mast cell calcium ion influx after a histamine-releasing stimulus. *Biochem Pharmacol* 25(5):505-510, 1976.

Sumner MJ, Cannon TR, Mundin JW, White DG, Watts DG: Endothelin ETA and ETB receptors mediate vascular smooth mzuscle contraction. *Br J Pharmacol* 107: 858-860, 1992.

Szabo 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* 8:284-291, 1997.

Takenaka T, Epstein M, Forster H, Landry DW, Iijima K, Goligorsky MS: Attenuation of endothelin effects by a chloride channel inhibitor, indanyloxyacetic acid. *Am J Physiol* 262(5Pt2):F799-806, 1992.

Tanaka T, Tsukuda E, Nozawa M, Nonaka H, Ohno T, Kase H, Yamada K, Matsuda Y.: RES-701-1, a novel, potent, endothelin type B receptor-selective antagonist of microbial origin. *Mol Pharmacol* 45(4):724-730, 1994.

Uchida Y, Ninomiya H, Sakamoto T, Lee JY, Endo T, Nomura A, Hasegawa S, Hirata F: ET-1 released histamine from guinea pig pulmonary but not peritoneal mast cells. *Biochem Biophys Res Commun* 189(2):1196-1201, 1992.

Valen G, Kaszaki J, Szabo I, Nagy S, Vaage J: release of histamine from isolated rat hearts during reperfusion is not dependent on length of ischemic insult, or contents of histamine in cardiac tissue. *Agents Actions* 40(1-2):37-43, 1993.

Valen G, Kaszaki J, Szabo I, Nagy S, Vaage J: Toxic oxygen metabolites and ischemia-reperfusion increase histamine synthesis and release in the isolated rat heart. *J Mol Cell Cardiol* 25(1):31-40, 1993.

Verburg KM, Bowsher RR, Henry DP: A new radioenzymatic assay for histamine using purified histamine N-methyltransferase. *Life Sci* 32:2855-2867, 1983.

Vural K.M., Liao H., Oz M.C., Pinsky D.J.: Effects of mast cell membrane stabilizing agent in a rat lung ischemia-reperfusion model. *Ann Thorac Surg* 69:228-232, 2000.

Warner TD, Allcock GH, Corder R, Vane JR: Use of endothelin antagonists BQ-123 and PD-142893 to reveal three endothelin receptors mediating smooth muscle contraction and release of EDRF. *Br J Pharmacol* 110:777-782, 1993.

White MV, Kaliner MA: Histamine. In Gallin JI, Goldstein IM, Snyderman R (eds): *"Inflammation: Basic principles and clinical correlates."* Raven Press, New York, p169, 1988.

Wolfard 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* 68(9):1231-1238, 1999.

Wolfard A, Kaszaki J, Szabo C, Szalay L, Nagy S, Boros M: Prevention of early myocardial depression in hyperdynamic endotoxemia in dogs. *Shock* 13(1):46-51, 2000.

Wypij DM, Nichols JS, Novak PJ, Stacy DL, Berman J, Wiseman JS: Role of mast cell chymase in the extracellular processing of big-endothelin-1 to endothelin-1 in the perfused rat lung. *Biochem Pharmacol* 43(4):845-853, 1992.

Yamamura H, Nabe T, Kohno S, Ohata K: Endothelin-1 induces release of histamine and leukotriene C4 from mouse bone marrow-derived mast cells. *Eur J Pharmacol* 257(3):235-242, 1994.

Yamamura H, Nabe T, Kohno S, Ohata K: Endothelin-1, one of the most potent histamine releasers in mouse peritoneal mast cells. *Eur J Pharmacol* 265(1-2):9-15, 1994.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki, T: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332(6163):411-415, 1988.

8. ANNEX