# ROLE OF ENDOTHELIN IN

# THE DEVELOPMENT OF MESENTERIC LOW-FLOW STATES

**Ph.D. Thesis**

**László Szalay M.D.**

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

**2003**

## INTRODUCTION

Mesenteric ischemia or ischemia-reperfusion is involved in several life-threatening pathologic conditions. The aim of our studies was the better understanding of these processes, thus contributing to further potential therapeutic regimens.

There is a growing body of evidence, that the release of endothelium-derived, potent vasoactive peptides, endothelins (ETs), play 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. The activated leukocytes, mast cells and other sources of inflammatory mediators then can modify the development of ischemia-reperfusion states significantly. Our aim was to observe the ET-induced leukocyte-and mast cell activation in the development of intestinal reperfusion injury.

We examined the impact of ET-A and B receptors on systemic macrohemodynamics, intestinal circulation, mast cell degranulation and granulocyte accumulation. Supposing the predominant role of the ET-A receptors, we then investigated the role of endogenous ET-A in changes of mesenteric macro- and microcirculatory parameters and leukocyte-endothelial cell interactions in a clinically relevant ischemia-reperfusion model. Finally, the effects of mast cells stabilization were examined on the ET-1-induced-leukocyte activation and macrohemodynamic alterations as well.

## MATERIALS AND METHODS

The experiments were performed on 108 rats (weighing 230±25 g), and 15 dogs (average weight: 15.4±4 kg)

**Examination of the systemic hemodynamic effects, mediated by ET-A and ET-B receptors**

Following a selective inhibition of ET-receptors, global circulatory response was induced by ET-1 infusion. We measured mean arterial pressure; the cardiac output was determined by the thermodilution method. Total peripheral resistance was derived from the previous parameters.

Experimental protocol: an ET-receptor antagonist (ET-A antagonists: BQ-610 or ETR-P1/fl; ET-B receptor blocker: IRL-1038) in a dose of 100 nmol/kg or its vehicle was infused into the systemic circulation over 10 min. An additional 10 min after the end of BQ-610, ETR-P1/fl or IRL-1038 peptide pretreatment, 1 nmol/kg ET-1 was infused i.v. over 10 min, and the circulatory effects were observed for another 60 min.

**Influence of ET-A receptor blockade on the mesenteric circulation and leukocyte activation following small bowel autotransplantation**

We registered the supplying blood flow and the local arterial/venous pressures of the intestinal graft; the segmental vascular resistance was calculated. The application of the orthogonal polarization spectral (OPS) imaging offered the measurement of the arteriolar and venular diameters, red blood cell velocities and functional capillary density without an infusion of any contrast material. The thickness of the epithelial layer was measured as an indicator of villus regeneration. The various phases of leukocyte-endothelial cell interactions were investigated by intravital fluorescent video microscopy (IVM). During reperfusion, the percentage of *rolling*, the number of *adherent* and *extravasated* cells was calculated.

Experimental protocol: the reanastomosed intestinal graft was reperfused after a 60 min cold ischemic period. In one group the ET-A receptor ETR-P1/fl peptide was infused (40nmol/kg) i.v. at the onset of reperfusion. To distinguish ET-A receptor mediated effects, a transplanted, but untreated group served as positive control. Morphologic, hemodynamic and intravital microscopic investigations of the transplanted intestine were performed through the 4 hrs reperfusion period.

**Investigation of the ET-1 mediated mast cell activation in the intestinal tract**

Intestinal perfusion (measured by laser-Doppler technique), tissue injury and mast cell degranulation were determined following ET-1 infusion. Mast cell degranulation and morphological alterations were determined from histological sections. Effects, mediated by different ET receptors were differentiated by the application of specific ET-A and ET-B antagonists.

Experimental protocol: in the first series of experiments we determined the dose-response relation of ET release and mast cell degranulation. In these groups, after the baseline measurements, a different doses of ET-1 (0.1, 1, 3 nmol/kg) were infused into the systemic circulation over 15 min to determine the relation of ET administration and mast cell degranulation. In an additional group of animals, segmental intestinal ischemia was induced by a 15-min occlusion of the ileal artery.

In the second series, 3 nmol/kg ET-1 was administered in combination with ET receptor blockers. In these groups, the ET-A receptor antagonists ETR-P1/fl and BQ-610 peptides, or the ET-B receptor antagonist IRL-1038 (300 nmol/kg) was infused i.v. for 30 min, followed by a 15-min of ET-1 infusion 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; finally, a tissue sample was taken from the intestinal segment then.

**Effects of ET-1 infusion on histamine release and leukocyte activation**

In this study we investigated the macrohemodynamics, intestinal histamine release and granulocyte accumulation following ET-1 infusion. The development of ET-mediated effects were influenced by ET-A receptor inhibition, mast cell degranulation was prevented by the mast cell stabilizer Na-cromoglycate. Cardiac output (thermodilution method) and mean arterial pressure were measured, total peripheral resistance was calculated. Histamine was determined from intestinal samples and plasma as well. We used tissue myeloperoxidase activity as a marker for granulocyte accumulation.

Experimental protocol: the ET-A receptor antagonist ETR-P1/f1 peptide (100 nmol/kg) or the MC stabilizer Na-cromoglycate (20 mg/kg) were infused i.v. for 10 min, followed by a 15-min of ET-1 infusion (1nmol/kg) into the systemic circulation after the end of ETR-P1/f1 peptide, or Na-cromoglycate pretreatment, respectively. The third group, receiving an identical dose of ET-1, but without any pretreatment served as positive control. After infusion, hemodynamic parameters were 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.

**Intravital video microscopy techniques**

*Orthogonal Polarization Spectral (OPS) imaging.* 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 tissue contribute to the images. This way, a virtual light source is created in tissues so those 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.

*Intravital fluorescent video microscopy (IVM).* The mesenteric microcirculation was analyzed using an epi-illumination technique. An ileal segment was gently placed on a specially designed stage. During the entire *in vivo* microscopic procedure the tissue was constantly bathed in 37 $^{0}$C Ringer's lactate to avoid drying and exposure to ambient air. Leukocytes were stained in vivo by means of rhodamine-6G.

The microscopic images were evaluated off-line from S-VHS video captures.

**Histology**

The damage of the intestinal mucosa was evaluated according to the Chiu grading system *(Arch. Surg. 101:478-483, 1970).*

Mucosal mast cells were examined following specific (alcian blue-safranin O) staining.

**Biochemical measurements**

Myeloperoxidase activity was determined according to the method of Kuebler et al. *(Int J Microcirc Clin Exp 16:89-97, 1996).*

Histamine assay was performed by means of a slightly modified version of the radioenzymatic method of Beaven and Horakova *(in Rocha e Silva M (ed):"Handbook of Experimental Pharmacology." Berlin: Springer-Verlag, 18:151, 1978).*

**RESULTS**

**The role of the single types of endothelin receptors in the exogenous endothelin-1-induced circulatory changes**

Infusion of 1 nmol/kg ET-1 induced an immediate rise in vascular resistance. The increase of arterial pressure was associated with a significant decrease in the cardiac output. The pretreatment with the ET-A receptor antagonist BQ-610 and ETR-P1/fl peptide significantly attenuated the ET-1-induced increase in the mean arterial pressure (MAP) and reduced the decrease in cardiac output (CO). ET-B receptor antagonist IRL-1038 peptide did not influence the ET-1-induced fall in CO. Similarly, the increase induced in the total peripheral resistance (TPR) by ET-1 was reduced by BQ-610 and ETR-P1/fl peptide pretreatment, whereas IRL-1038 was ineffective. The lack of influence of ET-B receptor blockade on the ET-1-induced hemodynamic alterations confirms the predominant role of ET-A receptors in the regulation of vascular tone.

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

Following transplantation, superior mesenteric artery flow (SMAF) decreased significantly in the non-treated group; the calculated mesenteric vascular resistance (MVR) values increased progressively. The ET-A receptor antagonist treatment significantly inhibited the transplantation-induced mesenteric hemodynamic changes. The infusion of ETR-P1/fl peptide significantly attenuated the fall in SMAF, and the rise in MVR. Villus functional capillary density (FCD) was significantly reduced in the untreated group, compared either to the baseline or the matching time point values. This reduction of FCD was significantly attenuated by the administration of ETR-P1/fl

peptide. Similarly, the ET-A receptor antagonist treatment also attenuated the tapering of the epithelial layer.

During reperfusion, 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 the ETR-P1/fl peptide treated-group. 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.

**ET-1-induced mast cell and leukocyte activation in the small intestine**

The infusion of increasing doses of ET-1 induced a dose dependent MC degranulation and mucosal injury in the rat small intestine. These effects could be ameliorated by ET-A receptor antagonist pretreatment, significantly. Blockade of ET-B receptors had no significant influence these alterations, however.

In agreement with our previous data, the inhibition of ET-A receptor mediated effects reduced both intestinal histamine release and leukocyte activation as well. Similarly, a mitigated histamine liberation and leukocyte deposition were observed following the administration of the mast cell stabilizing agent, Na-cromoglycate. The influence of leukocyte activation by Na-cromoglycate suggests a significant contribution of mast cells in the ET-mediated leukocyte activation.

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 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.

## 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 so much in learning the necessary surgical and experimental skills. I also express my thanks to all of my colleagues for their contributions to my experimental work.

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

## 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 autotransplantation: 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 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.