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Presence of Endogenous PACAP-38 Ameliorated Intestinal Cold Preservation Tissue Injury

Andrea Ferencz · Gyorgy Weber · Zsuzsanna Helyes · Hitoshi Hashimoto · Akemichi Baba · Dora Reglodi

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Abstract Cold preservation tissue injury remains an unsolved problem during small intestinal transplantation. Pituitary adenylate cyclase-activating polypeptide (PACAP) plays a central role in the intestinal physiology. The aim of our study was to compare the cold ischemic injury in wild-type and PACAP-38 deficient mice after small bowel cold storage. Cold ischemia was produced with small bowel preservation in a University of Wisconsin solution at 4°C in wild-type (n=35) mice for 1 h (GI), for 3 h (GII), and for 6 h (GIII); and in PACAP-38 deficient (n=35) mice for 1 h (GIV), for 3 h (GV), and for 6 h (GVI). Small bowel biopsies were collected after laparotomy (Control) and at the end of the ischemia periods. To determine oxidative stress parameters, malondialdehyde (MDA), reduced glutathione (GSH), and superoxide dismutase (SOD) were measured. Tissue damage was analyzed by qualitative and quantitative methods on hematoxylin/eosin-stained sections. In PACAP-38 deficient animals, tissue lipid peroxidation was elevated. These changes were significant after 6 h (153.04±7.2) compared to sham-operated (110.44±5.5) and compared to wild-type results (120.0±1.1 μmol/g, $p<0.05$). Meanwhile, the capacity and activity of the endogenous antioxidant system decreased significantly after 3 and 6 h preservation (GSH: 808.7±5.2; 720.4±8.7 vs. 910.4±μmol/g; SOD: 125.1±1.4; 103.3±1.9 vs. 212.11±5.8 IU/g). Qualitative and quantitative histological results showed destruction of the mucous, submucous layers, and crypts in PACAP-38 deficient mice compared to wild-type tissues. These processes depended on the time of the cold preservation periods. Our present study showed that the presence of PACAP-38 in the small bowel tissue has a key role in the protection against intestinal cold preservation injury.

Keywords Small bowel · Cold preservation · PACAP-38 · Oxidative stress · Tissue injury

A. Ferencz (✉) · G. Weber
Department of Surgical Research and Techniques,
University of Pécs Medical School,
Kodály Zoltán Street 20,
7624 Pecs, Hungary
e-mail: andrea.ferencz@aok.pte.hu

D. Reglodi
Department of Anatomy, University of Pecs,
Pecs, Hungary

Z. Helyes
Department of Pharmacology and Pharmacotherapy,
University of Pecs,
Pecs, Hungary

H. Hashimoto · A. Baba
Graduate School of Pharmaceutical Sciences, Osaka University,
Osaka, Japan

Introduction

Intestinal transplantation is inevitably accompanied by ischemia/reperfusion (I/R) injury. Although cold preservation is employed to reduce tissue degeneration, there is a progressive deterioration of cellular function over time (Ferencz et al. 2002, 2010). A number of parameters are used in addition to the histological picture as the "gold standard" to describe the injury (Fronek et al. 2006; Park et al. 1990). The solution most frequently used for the assessment of bowel graft preservation in clinical practice is the University of Wisconsin (UW) solution. In the background of preservation injury are several molecular processes, including production of oxygen-free radicals (OFRs), oxida-

tive stress, inflammatory processes, and apoptotic and necrotic cell death (Nedvig et al. 2009; Mittal et al. 2008).

Pituitary adenylate cyclase-activating polypeptide (PACAP) is widely distributed in peripheral organs including the gastrointestinal tract. The presence of PACAP has been shown in nerve elements as well as epithelial cells, glands, and blood vessel walls of the intestinal tissue (Koves et al. 1993; Vaudry et al. 2009). PACAP-38 exerts a wide variety of functions in the gastrointestinal system: it has effects on motility of the bowel and gallbladder wall, stimulates gastric acid secretion, induces hormone/enzyme release in the exocrine and endocrine pancreas and is involved in various gastrointestinal reflexes (Ekblad 1999; Ermilov et al. 2004; Kirchgessner and Liu 2001; Lauffer et al. 1999). PACAP-38 acts through specific, G-protein coupled receptors, the PAC1 receptors and VPAC1 and VPAC2 receptors that bind VIP and PACAP with equal affinity (Vaudry et al. 2009). All three types of PACAP receptors have been shown in the intestinal system: in the mucosa and myenteric neurons, in neuroendocrine cells, blood vessels, and smooth muscle (Mao et al. 1998; Salomon et al. 1993; Schulz et al. 2004).

PACAP-38 deficient mice provide an excellent tool to study the possible endogenous functions of PACAP (Hashimoto et al. 2001). Mice lacking endogenous PACAP-38 display several abnormalities in the nervous system and peripheral organs. In the intestinal system, it has been shown that mice lacking PACAP-38 react to dextran-induced colitis with a more severe functional and morphological outcome (Azuma et al. 2008; Nemetz et al. 2008). A recent study has reported that VIP-deficient mice also display several intestinal abnormalities (Lelievre et al. 2007). The aim of this study was to examine oxidative stress and tissue damage induced by different cold preservation periods in wild-type and PACAP-38 deficient mice.

Materials and Methods

Animals

Generation by a gene-targeting technique, maintenance, and backcrossing of CD1 PACAP deficient mice has been reported previously (Hashimoto et al. 2001). Mice were housed under pathogen-free conditions and were fasted for 24 h preoperatively, but had free access to water. Animals were anesthetized with intramuscular ketamine hydrochloride and diazepam (Richter Gedeon, Budapest, Hungary). All procedures were performed in accordance with the ethical guidelines of NIH and the guidelines approved by the University of Pecs (BA02/2000–20/2006) to minimize pain and suffering of the animals.

Cold Preservation Model

After median laparotomy, small bowel grafts were resected from the ligament of Treitz and stored in 4°C University of Wisconsin solution in wild-type mice for 1 h (Group I, $n=10$), for 3 h (Group II, $n=10$), and for 6 h (Group III, $n=10$); and in PACAP-38 deficient mice for 1 h (Group IV, $n=10$), for 3 h (Group V, $n=10$), and for 6 h (Group VI, $n=10$). Sham-operated animals ($n=10$) underwent median laparotomy only. Intestinal biopsies were collected after laparotomy (Control) and at the end of the ischemic periods.

Biochemical Assays

Oxidative stress parameters were measured as previously described (Ferencz et al. 2009a). Briefly, malondialdehyde (MDA) was determined in bowel tissue homogenates with the use of a lipid peroxidation assay kit, a colorimetric assay kit specific for MDA. Final values were given as micromoles per gram. Reduced glutathione (GSH) was quantified in bowel tissue homogenates using a glutathione assay kit. This method allows transforming GSH into a chromophoric thione with a maximal absorbance at 400 nm. Values of glutathione were expressed in micromoles per gram. Superoxide dismutase (SOD) was measured in bowel tissue homogenates using a superoxide dismutase assay kit. One reagent of the kit underwent alkaline auto-oxidation, which was accelerated by SOD. Auto-oxidation of this reagent yielded a chromophore, which absorbed maximally at 525 nm. The value of the activity of SOD was given in units per gram. All assay kits were obtained from Calbiochem-Novabiochem Corp, Darmstadt, Germany.

Histology

Bowel tissues were fixed in 10% buffered formalin, embedded, cut to 10 μm, and stained with hematoxylin and eosin. Histologic damage was assessed in a 'blind' manner (D.R. and A.F.) using Park's histologic classification of intestinal injury (Nikon Eclipse 80 Light Microscope, Kingston, England) (original magnification ×100) (Park et al. 1990). Total mucosa and submucosa thickness, depth of the crypts, and thickness of muscular layer were quantified using the software Scion Image (Scion Corporation, Maryland, USA). The number of square pixels was counted in five fields per sections at ×400 magnification, and the length was given in micrometers.

Statistics

Results are expressed as mean values±SEM. Data were analyzed with one-way analysis of variance (ANOVA). The level of significance was set at $P<0.05$. The MicroCal

Origin (ver. 6.0) program (Microcal Software Inc, Northampton, USA) was used for data evaluation.

Results

Biochemical Results

The extent of lipid peroxidation was determined by measuring the tissue concentration of its byproduct, malondialdehyde. Cold intestinal preservation increased the concentration of tissue MDA in each group in a time-dependent manner compared to the control and Sham groups. Significant elevation was measured in wild-type and in PACAP-38 deficient tissues after 3 and 6 h cold storage compared to control. Moreover, significant difference of MDA value was between wild-type (GII) and PACAP-38 deficient mice (GV) following 3 h ischemia; and between wild-type (GIII) and PACAP-38 deficient mice (GVI) following 6 h preservation (Fig. 1).

The content of the endogenous scavenger GSH decreased in all groups compared to control. However, GSH value decreased significantly in PACAP-38 deficient groups after 3 and 6 h ischemia (GV–GVI) compared to the control and Sham groups. Moreover, GSH levels were significantly different between GIII and GVI (Fig. 2).

SOD activity decreased in each group, these changes were significant in GII–GIII and in GV–GVI compared to control. Furthermore, better preservation of SOD activity was observed in wild-type groups (GII and GIII), than in PACAP-38 deficient tissues (GV and GVI). It was significantly higher in GIII, than in GVI (Fig. 3).

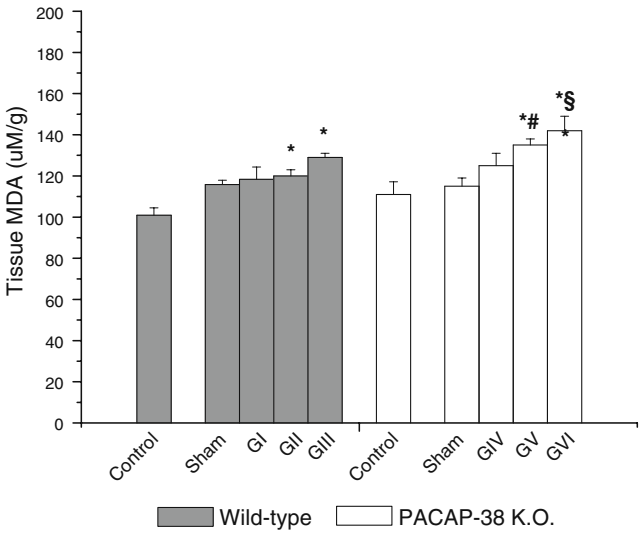

Figure 1 Changes of tissue malondialdehyde following intestinal cold preservation. Small bowel was stored in UW solution in wild-type mice for 1 h (GI), for 3 h (GII), and for 6 h (GIII); and in PACAP-38 deficient mice for 1 h (GIV), for 3 h (GV), and for 6 h (GVI). Biopsies were collected after laparotomy (Control), and at the end of the reperfusion periods. Final value of MDA was given as μmol/g. Data are presented as mean±SEM. *P<0.05 vs. Control; #P<0.05 vs. GII; §P<0.05 vs. GIII

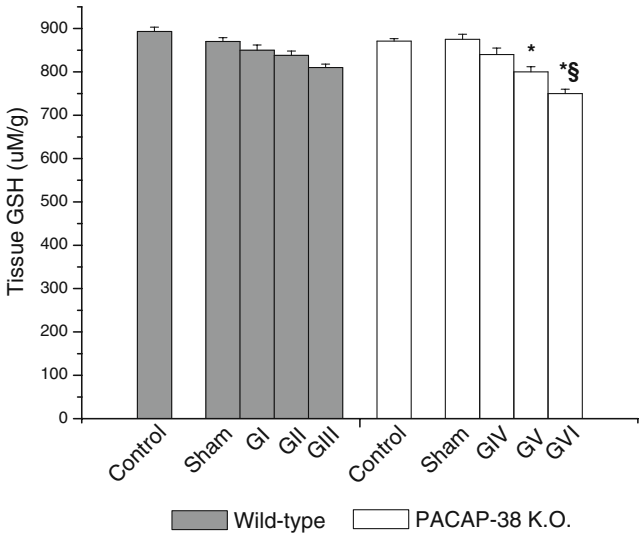

Figure 2 Changes of tissue-reduced glutathione following intestinal cold preservation. Small bowel was stored in UW solution in wild-type mice for 1 h (GI), for 3 h (GII), and for 6 h (GIII); and in PACAP-38 deficient mice for 1 h (GIV), for 3 h (GV), and for 6 h (GVI). Biopsies were collected after laparotomy (Control), and at the end of the reperfusion periods. Final value of GSH was given as μmol/g. Data are presented as mean±SEM. *P<0.05 vs. Control; §P<0.05 vs. GIII

Histology

According to Park's classification, the highest grade of injury was observed in PACAP-38 deficient tissue after 6 h cold storage (GVI), whereas the lowest grade of injury was found in wild-type tissue following 1 h preservation (GI). The sham group showed an injury grade 0, corresponding to normal bowel structure. After 1 h cold preservation in wild-type tissue (GI), the histological findings were corresponding to an injury grade 1, showing minor clefting with the villus epithelium adjacent to the crypts intact. In contrast, in PACAP-38 deficient tissue (GIV) the structural injury was grade 2, with subepithelial space at villus tip and more villus clefting. Three hours cold ischemia in wild-type intestine showed an injury of grade 2 only. These signs were more severe in PACAP-38 deficient tissue, where the histological analyses showed an injury of grade 3, characterized by massive epithelial lifting and villus tip denudation (GV). In wild-type tissue, 6 h cold preservation resulted in injury of grade 3 (GIII). In PACAP-38 deficient mice, 6-h cold storage-caused injury showed severely injured crypts and denuded villi corresponding to a grade 4 injury (Fig. 4).

By Scion Image quantitative analysis, mucosal thickness decreased significantly in GII–GVI compared to control.

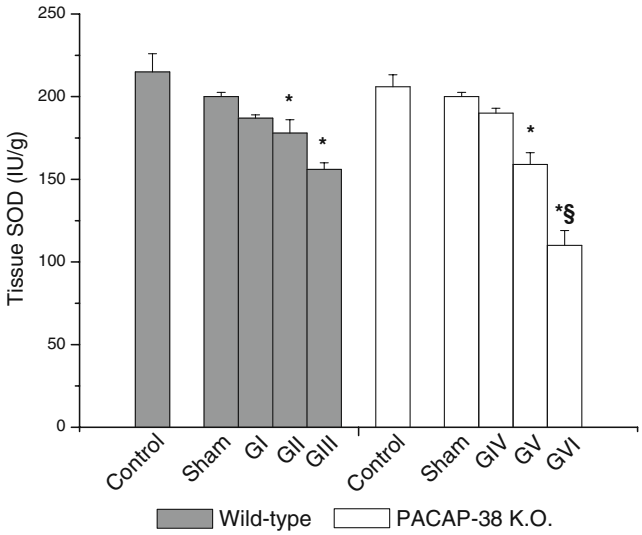

Figure 3 Changes of tissue superoxide dismutase following intestinal cold preservation. Small bowel was stored in UW solution in wild-type mice for 1 h (GI), for 3 h (GII), and for 6 h (GIII); and in PACAP-38 deficient mice for 1 h (GIV), for 3 h (GV), and for 6 h (GVI). Biopsies were collected after laparotomy (Control), and at the end of the reperfusion periods. Final value of SOD activity was given in IU/g. Data are presented as mean±SEM. *P<0.05 vs. Control; §P<0.05 vs. GIII

Significant difference was between GI and GIV; and between GIII and GVI, showing better tissue preservation in wild-type, than in PACAP-38 deficient intestine (Fig. 5a). Submucosal thickness decreased significantly in GIII–GVI compared to control (Fig. 5b). Depth of crypts decreased significantly in GVI tissues (Fig. 5c). Muscle thickness showed mild decrease in each group compared to controls, but these changes were not significantly different by the end of the preservation periods (Fig. 5d).

Discussion

This study examined the oxidative stress parameters and degree of tissue injury following intestinal cold preservation on wild-type and PACAP-38 deficient mice.

Preservation graft injury is a phenomenon associated with each organ transplant procedure, and, to a certain extent, affects adversely graft quality. The presence of I/R injury and its impact on the small bowel graft contribute to the development of postoperative complications. These include primary graft non- or dysfunction, endotoxemia, peritonitis, and risk of acute/chronic rejection. Given the negative effect of this phenomenon, efforts are being made to recognize, modulate, minimize, or even eliminate it (Carden and Granger 2000; Ferencz et al. 2009b; Balaz et al. 2004; Mallick et al. 2004, 2005).

In this study, the effect of cold preservation is defined by the development of oxidative stress in small bowel tissue. Our results with homogenates showed that value of lipid peroxidation elevated in a time-dependent manner, but it was significantly higher in PACAP-38 deficient than in

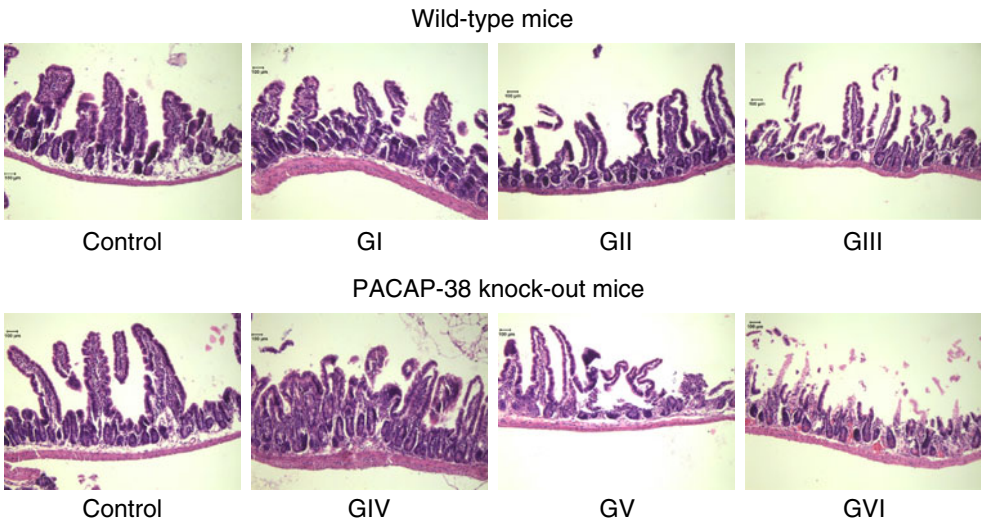

Figure 4 Tissue injury of small bowel after cold storage. Intestine was preserved in UW in wild-type mice for 1 h (GI), for 3 h (GII), and for 6 h (GIII); and in PACAP-38 deficient mice for 1 h (GIV), for 3 h (GV), and for 6 h (GVI). Biopsies were collected after laparotomy (Control), and at the end of the reperfusion periods. Park's classification was used as the standard grading system (grades 0 to 5). The sham group showed an injury grade 0, corresponding to normal bowel structure. *GI* representative of grade 1 injury, showing minor clefting with the villus epithelium adjacent to the crypts intact. *GII* representative of grade 2 injury with subepithelial space at villus tip and more villus clefting. *GIII* representative of grade 3 injury with massive epithelial lifting and villus tip denudation. *GIV* representative of grade 2 injury with subepithelial space at villus tip and more villus clefting. *GV* representative of grade 3 injury with massive epithelial lifting and villus tip denudation. *GVI* representative of grade 4 injury with severely injured crypts and denuded villi. (Hematoxylin and eosin staining, original magnification ×100). Bar=100 μm

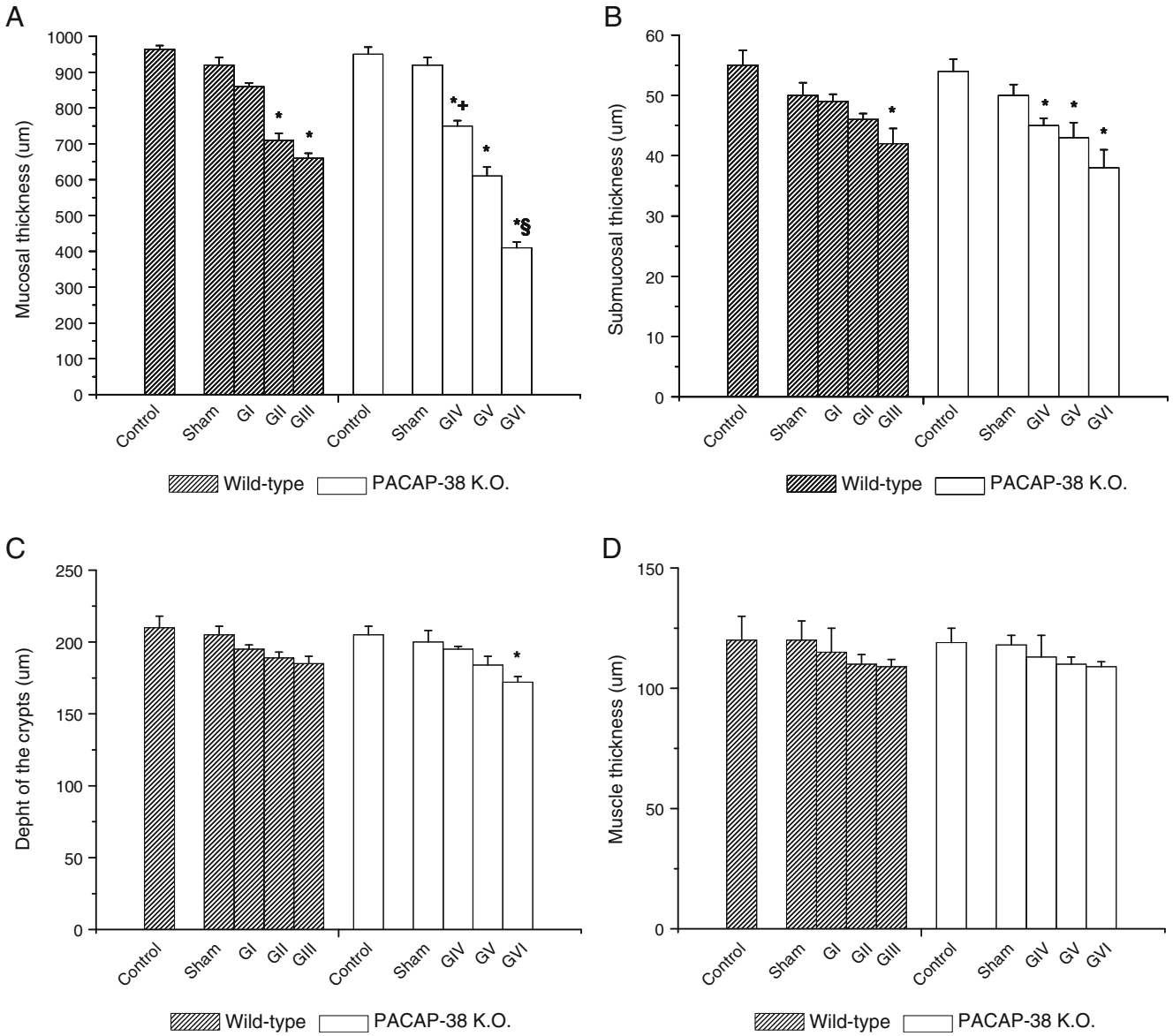

Figure 5 Quantitative analysis of tissue injury of small bowel following cold preservation. Cold storage in UW was in wild-type mice for 1 h (GI), for 3 h (GII), and for 6 h (GIII); and in PACAP-38 deficient mice for 1 h (GIV), for 3 h (GV), and for 6 h (GVI). Biopsies were collected after laparotomy (Control), and at the end of the reperfusion periods. Final measures of intestinal structures were given in μm. Data are presented as mean±SEM. **a** Mucosal thickness; **b** Submucosal thickness; **c** Depth of the crypts; **d** Muscle thickness. *$P<0.05$ vs. Control; §$P<0.05$ vs. GIII

wild-type animals. Tissue levels of endogenous antioxidant scavenger GSH decreased both in wild-type and PACAP-38 deficient mice during cold storage, its concentration significantly decreased in PACAP-38 deficient intestine after 3 and 6 h. The activity of endogenous antioxidant SOD decreased significantly following 3 and 6 h cold storage both in wild-type and in PACAP-38 deficient tissues. Moreover, in wild-type intestine, the activity remained to a greater extent, and it was significantly higher than in PACAP-38 deficient tissues. There are no data indicating the exact mechanism of the protective effect of PACAP-38 in the early phase in ischemic small bowel. Several studies show, however, that the antioxidant, anti-apoptotic, and anti-inflammatory effects of the peptide are important in its cytoprotective effects (Reglodi et al. 2004; Somogyvari-Vigh and Reglodi 2004; Vaudry et al. 2009). PACAP has mild direct scavenger activity, but it is suggested that PACAP rather acts indirectly, via stimulating antioxidant enzyme activity/synthesis and via influencing the oxidative stress-induced pathways (Reglodi et al. 2004). As far as the oxidative stress-induced signaling pathways are concerned, PACAP has been shown to counteract the

alteration caused by oxidative stress at several levels of the downstream pathway in various cells of different origin (Racz et al. 2007; Vaudry et al. 2002).

The present observations are in accordance with our previous observations, where we have shown that adding exogenous PACAP-38 to UW solution prevented the elevation of lipid peroxidation and the reduction in endogenous scavenger capacity in rat small intestine. The intestinal structure was also significantly better preserved in small bowel autotransplantation model when PACAP-38 was added to the preservation solution (Ferencz et al. 2009b). These observations support the role of PACAP-38 in tissue preservation in cold preservation model of transplantation. Moreover, we have also shown that endogenous PACAP-38 significantly decreased during intestinal cold preservation and autotransplantation (Ferencz et al. 2009b, 2009c). This shows that endogenous PACAP-38 sensitively reacts to intestinal ischemic stimuli, which may induce further damage.

In the present study we also demonstrated that cold preservation caused tissue injury in the small bowel. This damage correlated with the duration of preservation time, with the highest destruction observed in tissue following 6-hour storage in PACAP-38 deficient mice. Both qualitative and quantitative analyses in our study demonstrated that PACAP-38 ameliorated tissue injury induced by cold storage. Mucosal and submucosal thickness, and depth of crypts were better preserved in tissues containing PACAP-38 in wild-type animals. The exact mechanism of the protective effect of PACAP-38 in the present model is not yet known.

Our present study is also in accordance with the numerous reports that demonstrate different abnormalities in PACAP-38 deficient mice. Among others, it has been shown that mice lacking PACAP-38 display altered cerebellar development and abnormal axonal arborization (Allais et al. 2007; Yamada et al. 2010). Interestingly, in peripheral tissues, which have been studied in PACAP-38 deficient mice, intact tissues show little or no alteration compared to wild-type animals. This has been also described in the large intestine (Azuma et al. 2008; Nemetz et al. 2008). However, when tissues of different origin are exposed to harmful stimuli, PACAP-38 deficient mice react with a significantly worse outcome in most tested models. This has been found in dextran-induced colitis model (Azuma et al. 2008), in cerebellar granule cells and kidney cell culture exposed to oxidative stress (Horvath et al. 2010; Vaudry et al. 2005), in cerebral ischemia (Ohtaki et al. 2008), and in axonal regeneration (Armstrong et al. 2008). We also found that intact small intestinal structure was not different between wild-type and PACAP-38 deficient mice, but these latter animals reacted to injury with a worse outcome. An intact intestinal mucosa is of vital importance for efficient assimilation of ingested nutrients, but it also serves as a barrier that limits access of enteric bacteria and other noxious stimuli to the systemic circulation.

In summary, our present results support the protective role of endogenous PACAP-38 in cold preservation of small intestine, which may have clinical relevance in bowel transplantation in the future.

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