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Intestinal Autotransplantation Using PACAP-38-Containing Preservation Solution

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Abstract Small bowel is one of the most sensitive organs to ischemia–reperfusion injury, which is a significant problem during transplantation. Pituitary adenylate cyclase-activating polypeptide (PACAP) has cytoprotective effect in ischemic injuries of various tissues. The aim of our study was to measure changes of PACAP-38 and PACAP-27 immunoreactivities and cytokine levels in intestinal grafts stored in PACAP-38-containing preservation solution. Small bowel autotransplantation was performed on male Wistar rats. Grafts were stored in University of Wisconsin (UW) solution at 4 °C for 1 h (group (G)I), for 3 h (GII), and for 6 h (GIII) and in PACAP-38-containing UW solution for 1 h (GIV), for 3 h (GV), and for 6 h (GVI). After preservation, performing vessel anastomosis reperfusion began, which lasted 3 h in each group. Tissue biopsies were collected after laparotomy (control) and at the end of the reperfusion periods. Intestinal PACAP-38 and PACAP-27 immunoreactivities were measured by radioimmunoassay. To measure cytokine array from tissue homogenates, we used rat cytokine array and Luminex Multiplex Immunoassay. Levels of PACAP-38 and PACAP-27 immunoreactivity decreased after 1 and 3 h preservation compared to control levels. This decrease was significant following 6 h cold storage ($p<0.05$). Values remained significantly higher in grafts stored in PACAP-38-containing UW. Cytokine array revealed that expression of the soluble intercellular adhesion molecule-1 (CD54) and L-selectin (CD62L/LECAM-1) was increased in GIII. Both 6 h cold storage in PACAP-38-containing UW solution and 3 h reperfusion caused strong reduction in these cytokines activation in GVI. RANTES (CCL5) levels were increased in all groups. Strong activation of the tissue inhibitor of metalloproteinase-1 was in GIII. However, PACAP-38-containing cold storage could decrease its activation in GVI. Furthermore, strong activation of the tissue inhibitor of metalloproteinase-1 was detected in 6 h preserved grafts without PACAP-38 (GIII). PACAP-38-containing cold storage could decrease its activation in GVI. Our present study showed that PACAP-38 and PACAP-27 immunoreactivities decreased in a time-dependent manner during intestinal cold preservation, which could be ameliorated by administration of exogenous PACAP-38 to the preservation solution. Moreover, PACAP-38 could attenuate tissue cold ischemic injury-induced changes in cytokine expression.

Keywords Small bowel · Transplantation · PACAP-38 · PACAP-27 · Cytokine

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Introduction

Small bowel is a highly sensitive tissue to ischemia/reperfusion (I/R) injury in the body. Intestinal I/R injury is caused by many clinical conditions, including small bowel transplantation. Both clinical and experimental data demonstrate that transplant I/R injury has deleterious short- and long-term effects, manifesting as increased episodes of acute rejection and chronic allograft dysfunction (Ferencz et al. 2002, 2010a, b; Linfert et al. 2009; Yuan et al. 2011). Graft viability prior to implantation is a key factor in the outcome after organ transplantation. Along with surgical manipulation, I/R injury and preservation damage are some of the many essential factors that affect the quality of intestinal graft and its multiple functions. The current standard in organ preservation with University of Wisconsin (UW) solution was developed for kidney/liver preservation and it is suboptimal for the intestinal graft despite good results for other organs (Maathuis et al. 2007; Roskott et al. 2011). The benefit of the UW solution for the preservation of other intraabdominal organs remains unclear and the maximum storage time for small bowel remains relatively brief (6–8 h). Thus, no general agreement exists about optimal preservation solution for intestinal grafts so far (Kokotilo et al. 2010; Roskott et al. 2011). Recently, there are continuous research efforts to modify the commercially available solutions (adding more components, high-energy intermediates, and nutrients) or to develop new preservation solutions (Inuzuka et al. 2007; Wei et al. 2007; Ferencz et al. 2009; Yandza et al. 2011).

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a widespread neuropeptide with diverse effects not only in the nervous system but also in the cardiovascular system and peripheral organs including endocrine glands, respiratory organs, and gastrointestinal tract. The polypeptide exists in two forms, with 38 and 27 amino acid residues, named PACAP-38 and PACAP-27 (Vaudry et al. 2009). Endogenous PACAP-38 and PACAP-27 were demonstrated in all parts of the gastrointestinal tract with high levels detected in the jejunum and ileum. PACAP-38 and PACAP-27-immunoreactivities have been shown in the cell bodies and nerve fibers throughout the gastrointestinal tract (Hannibal et al. 1998). PACAP-38 and PACAP-27 act through the specific PAC1 receptor and VPAC1 and VPAC2 receptors that bind VIP and PACAP with equal affinity (Koves et al. 1993; 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 (Schulz et al. 2004; Pirone et al. 2011).

Endogenous PACAP-38 has been implicated in protection against harmful stimuli; the peptide has anti-apoptotic, anti-inflammatory, and anti-oxidant actions in numerous different in vivo and in vitro models (Ferencz et al. 2010a; Horvath et al. 2010a; Gasz et al. 2006; Racz et al. 2006, 2010; Reglodi et al. 2011, 2012; Roth et al. 2009). Recently, in warm and cold I/R small bowel models, the in vivo protective effects of PACAP-38 have been shown. These experiments confirmed an important protective effect of endogenous PACAP-38 against warm I/R tissue damage. Moreover, it has been shown that adding exogenous PACAP-38 to UW solution prevented the oxidative stress and tissue structure injury in rat small intestine (Ferencz et al. 2010a, b). Although the exact mechanisms, by which intestinal I/R and gut injury contribute to the systemic inflammatory response, are not completely known, cytokines play a key role in these actions. There is also evidence that intestinal injury resulting from I/R can lead to the bowel becoming a cytokine-generating organ (Grotz et al. 1999). The anti-inflammatory actions of PACAP in several inflammatory models are partially mediated through its suppressing effect on cytokine/chemokine production (Delgado and Ganea 2001; Horvath et al. 2010b; Ohtaki et al. 2010). However, there are no data in the literature about changes of intestinal PACAP-38 or PACAP-27 and tissue cytokine levels using PACAP-38-containing preservation solution in stored and transplanted small bowel grafts. The aim of our study was to measure changes of PACAP-38 and PACAP-27 immunoreactivities and cytokine values using PACAP-38-containing UW preservation solution during intestinal autotransplantation.

Materials and Methods

Animals

Adult male Wistar rats (250–300 g, n=35) were purchased from the Laboratory Animal Center of University of Pecs, housed under pathogen-free conditions, and were fasted for 24 h preoperatively but had free access to water. Rats were anesthetized with intramuscular ketamine hydrochloride (0.075 mg/g of body weight) and diazepam (0.075 mg/g of body weight; Richter Gedeon, Budapest, Hungary). All procedures were performed in accordance with the ethical guidelines of National Institute of Health and guidelines approved by the University of Pecs (BA02/2000-9/2008) to minimize pain and suffering of the animals.

Intestinal Autotransplantation Model

Rats were randomly divided into groups (G). In group S, animals underwent only median laparotomy (sham operated, n=5). In GI–GVI after heparin administration (0.2 U/g), small bowel was resected to descending colon, and the lumen was flushed with normal saline. Grafts were perfused

by the superior mesenteric artery and were preserved in 100 ml 4 °C University of Wisconsin solution (ViaSpan, DuPont Pharma, Bad Homburg, Germany) for 1 h in GI (n=5), for 3 h in GII (n=5), and for 6 h in GIII (n=5). Grafts were preserved in 100 ml UW solution containing 100 μg PACAP-38 dissolved in 2 ml of physiological saline (Sigma, Hungary) for 1 h in GIV (n=5), for 3 h in GV (n=5), and for 6 h in GVI (n=5). After preservation, end-to-end anastomosis was performed between the stumps of mesenteric vessels with microvascular technique. Reperfusion lasted 3 h in each group. Small bowel biopsies were collected after laparotomy (control, C) and at the end of the reperfusion periods.

Radioimmunoassay

Intestinal tissue samples (600 mg) were homogenized in ice-cold distilled water. After centrifugation at 12,000 rpm/4 °C for 30 min, the supernate was further processed for RIA analysis of PACAP-38- and PACAP-27-like immunoreactivity, as previously described (Brubel et al. 2011). Briefly, the antiserum for PACAP-38 was "88111-3" and for PACAP-27 was "88123". The tracer was mono-^{125}I-labeled ovine PACAP 24-38 and mono-^{125}I-labeled ovine PACAP-27 (5,000 cpm/tube). The standard was ovine PACAP-38 and PACAP-27 (0–1,000 fmol/ml). Assays were prepared in 1 ml phosphate buffer (0.05 mol/l, pH 7.4) containing 0.1 M NaCl, 0.05 % NaN_3, and 0.25 % bovine serum albumin. The assay procedures include 100 μl antiserums (working dilutions—PACAP-38 "88111-3" antiserum, 1:10,000; PACAP-27 "88123" antiserum, 1:45,000), 100 μl RIA tracers, and 100 μl standards or unknown samples measured into polypropylene tubes with assay buffer. After 48–72 h incubation at 4 °C, the antibody-bound peptides were separated from the free ones by addition of 100 μl separation solution (10 g charcoal, 1 g dextran, and 0.2 g commercial fat-free milk powder in 100 ml distilled water). Following centrifugation (3,000 rpm for 20 min at 4 °C), the tubes were gently decanted, and the radioactivity of the precipitates was measured in a gamma counter. PACAP-38 and PACAP-27 concentrations of the unknown samples were read from the appropriate calibration curves. Results of PACAP-38- and PACAP-27-like immunoreactivities are given as femtomoles per milligram tissue.

Cytokine Array After Small Bowel Autotransplantation

Intestinal tissues from control bowel sample (A), from tissue exposed to 6 h cold storage in UW (B), from 6 h cold preservation in PACAP-38-containing UW solution (C), and subsequent 3 h reperfusion period (D) were measured as previously described (Horvath et al. 2011). Briefly, cytokine array from tissue homogenates was performed using rat cytokine array (Panel A Array kit from R&D Systems, Biomedica Hung., Budapest, Hungary). Small bowel samples were excised then homogenized in PBS with protease inhibitors. Triton X-100 was added to the final concentrations of 1 %. The samples were stored at −80 °C prior to use. After blocking the array membranes for 1 h and adding the reconstituted Detection Antibody Cocktail for another 1 h at room temperature, the membranes were incubated with 1 ml of tissue homogenates at 2–8 °C overnight on a rocking platform. After washing with buffer for three times and addition of horseradish peroxidase-conjugated streptavidin to each membrane, we exposed them to a chemiluminescent detection reagent (Amersham Biosciences, Hungary) then side up to an X-ray film cassette.

Luminex Multiplex Immunoassay

The levels of three host markers (soluble intercellular adhesion molecule-1 (sICAM1), L-selectin, and metalloproteinase-1 (TIMP-1)) were determined in the selected bowel samples (see in cytokine array) using customized Flurokine MAP Rat Base Kit (R&D Systems). This was done according to the manufacturer's instructions (R&D Systems). Following previous optimizations, all samples were tested undiluted, in a blinded manner. All analyte levels in the quality control reagents of the kits were within the expected ranges. Standard curve for sICAM-1 is 17–12,500 pg/ml, for L-selectin is 100–73,000 pg/ml, and for TIMP-1 is 55–40,600 pg/ml. Measurements were done with Luminex100 instrument, and Luminex 100 IS software was used for the analysis of bead median fluorescence intensity. The R&D Systems Rat Base kit assay was carried out according to the manufacturer's instructions, with a few exceptions as stipulated below. Briefly, an eight-point standard curve was generated by performing serial dilutions of the reconstituted normalized standard (lot # 1279612). This was done in order to ensure that the matrix used in the generation of the standard curve resembled that of the samples as closely as possible as preliminary test showed that this method was superior to dilution of standards in standard diluent (data not shown). Bowel samples were homogenized with RPMI-1640 (GIBCO) containing 1 % protease inhibitor cocktail. In order to assess recovery, bowel samples were used in 20 mg/ml concentrations. The assays were run in duplicate, which produced in total of six concentration replicates. A 50-μl volume of each sample, control, or standard was added to a 96-well plate (provided with the kit) containing 50 μl of antibody-coated fluorescent beads. Biotinylated secondary and streptavidin-PE antibodies were added to the plate with alternate incubation and washing steps. After the last wash step, 100 μl of wash buffer was added to the wells; the plate was incubated and read on the Luminex100 array reader, using a four-PL regression curve to plot the standard curve. Data were subsequently analyzed using the Luminex100 manager software.

Statistics

Results are expressed as mean values ± SEM. Data were analyzed with one-way analysis of variance. 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

Radioimmunoassay

Level of intestinal PACAP-38-like immunoreactivity (LI) was 55.1±2.5 fmol/mg in sham-operated group and it was 57.32± 3.5 fmol/mg in control samples. After 1 h cold storage, intestinal PACAP-38-LI was 50.4±3.5 fmol/mg (GI), and after 3 h preservation it decreased to 40.1±5.5 fmol/mg (GII). These changes were significant following 6 h cold storage (GIII, 32.6±3.0 fmol/mg; $p<0.05$) compared to control or sham values. Levels remained significantly higher in grafts stored in PACAP-38-containing UW solution (GIV–GVI). In GIV, levels (65.2±3.4 fmol/mg) increased above the control values, which was statistically significant. After 3 and 6 h cold storage in PACAP-38-containing preservation solution, the PACAP-38-LI levels were 55.6±4.2 fmol/mg (GV) and 48.9± 3.2 fmol/mg (GVI). These resulted significantly higher compared to preservation only in UW without PACAP-38 (vs. GII and GIII; $p<0.05$) (Fig. 1).

Tissue PACAP-27 level also decreased during cold storage and autotransplantation procedure compared to the control value (4.2±0.2 fmol/mg). This decrease was significant in the 1 h (GI, 2±0.2 fmol/mg; $p<0.05$), 3 h (GII, 1.6± 0.3 fmol/mg; $p<0.05$), and 6 h (GIII, 0.9±0.2 fmol/mg; $p<0.01$) groups. Levels of PACAP-27-LI remained significantly higher in grafts stored in PACAP-38-containing UW solution (GIV–GVI) at the end of the reperfusion periods. In GIV, its concentration was 3.5±0.3 fmol/mg, which was significantly higher than in GI. Three and 6 h cold storage in PACAP-38-containing preservation solution resulted in elevated PACAP-27 levels in GV (3.0±0.2 fmol/mg) and in GVI (2.6±0.15 fmol/mg). These values were significantly higher compared to tissues preserved in UW without PACAP-38 (vs. GII and GIII; $p<0.05$) (Fig. 2).

Cytokine Measurements

Among several cytokines, according to cytokine array, the expression of the sICAM-1 (CD54) (1) and L-selectin (CD62L/LECAM-1) (2) regulated upon activation was detectable in control bowel samples. The expression did not changed after 6 h cold preservation in UW and subsequent reperfusion period in GIII. Both 6 h cold storage in PACAP-38-containing UW solution and 3 h reperfusion caused a strong reduction in the activation of these cytokines in GVI. The RANTES (CCL5) (3) levels were high in all groups and did not change, as could be observed in the

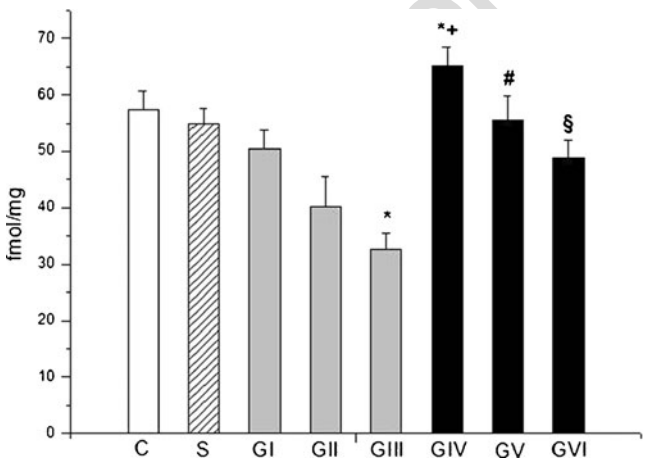

Fig. 1 Level of immunoreactive PACAP-38 in small bowel tissue after intestinal autotransplantation. Small intestinal grafts were perfused and preserved in cold UW solution for 1 h (GI), for 3 h (GII), and for 6 h (GIII) and in UW containing PACAP-38 for 1 h (GIV), for 3 h (GV), and for 6 h (GVI). In sham-operated group (S), animals underwent only median laparotomy. Small bowel biopsies were collected after laparotomy (control, C) and at the end of the reperfusion periods. Final value of PACAP-38 was given as femtomoles per milligram wet weight. Data are presented as mean ± SEM. $^{*}p<0.05$ vs. control; $^{+}p<0.05$ vs. GI; $^{\#}p<0.05$ vs. GII; $^{\S}p<0.05$ vs. GIII

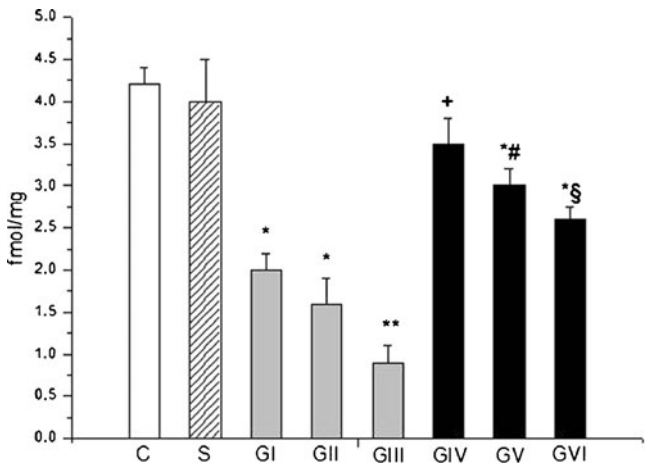

Fig. 2 Level of immunoreactive PACAP-27 in small bowel tissue during autotransplantation. Small intestinal grafts were perfused and preserved in cold UW solution for 1 h (GI), for 3 h (GII), and for 6 h (GIII) and in UW containing PACAP-38 for 1 h (GIV), for 3 h (GV), and for 6 h (GVI). In sham-operated group (S), animals underwent only median laparotomy. Small bowel biopsies were collected after laparotomy (control, C) and at the end of the reperfusion periods. Final value of PACAP-27 was given as femtomoles per milligram wet weight. Data are presented as mean ± SEM. $^{*}p<0.05$ vs. control; $^{**}p<0.01$ vs. control; $^{+}p<0.05$ vs. GI; $^{\#}p<0.05$ vs. GII; $^{\S}p<0.05$ vs. GIII

PACAP-treated groups. We found no activation of the tissue inhibitor of TIMP-1 (4) in the control samples, but strong activation was detected in 6 h preserved grafts without PACAP-38 (GIII). PACAP-38-containing cold storage could decrease its activation in GVI (Fig. 3).

Measurement of cytokines levels by Luminex Immunoassay confirmed these results (Fig. 4a–c). sICAM and L-selectin were expressed at similar levels in the control (A in Fig. 4a, b) and ischemic groups (B in Fig. 4a, b), while both were significantly reduced in the PACAP-treated groups (C and D in Fig. 4a, b). TIMP, on the other hand, was expressed at detection limit in the control group (A in Fig. 4c), and it was markedly increased upon ischemia (B in Fig. 4c). The elevated TIMP levels were significantly attenuated by PACAP treatment (C and D in Fig. 4c).

Discussion

This study examined the intestinal levels of PACAP-38 and PACAP-27 and tissue cytokine expression using PACAP-38-containing UW preservation solution during small bowel autotransplantation.

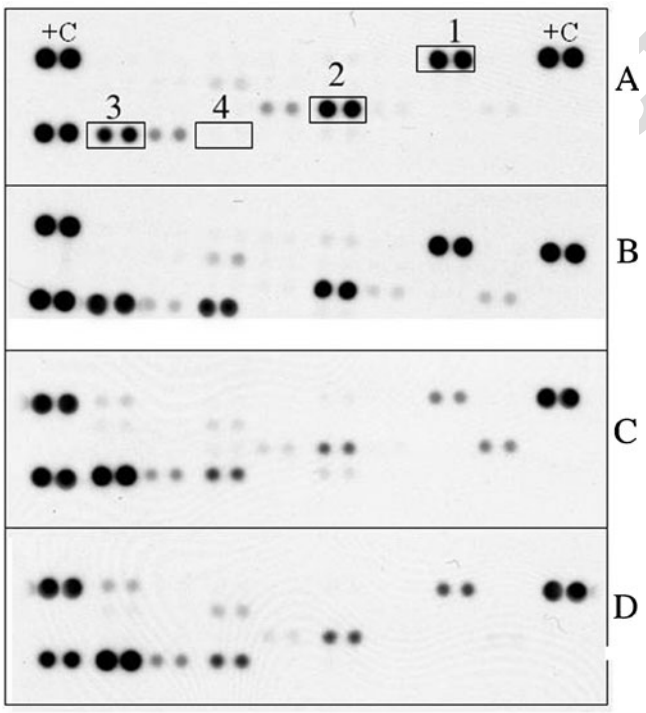

Fig. 3 Cytokine array showing the appearance on various cytokines in control intestine (**a**) and in small bowel tissue exposed 6 h cold storage in UW (**b**) or 6 h cold preservation in PACAP-38-containing UW solution (**c**) and subsequent 3 h reperfusion period (**d**). Marked changes are observed in sICAM-1 (*1*), L-selectin (*2*), and TIMP-1 (*4*) expressions. *+C* represents positive control. Other spots, where no changes were observed are (from *upper left corner*, *without numbers*): CINC-1, CINC-2alpha/beta, CINC-3, GM-CSF, IFN-gamma, IL-1alpha, IL-1beta, IL-1ra, IL-2, IL-3, IL-4, IL-6, IL-10, IL-13, IL-17, IP-10, LIX, MIG, MIP-1alpha, TNF-alpha, and VEGF

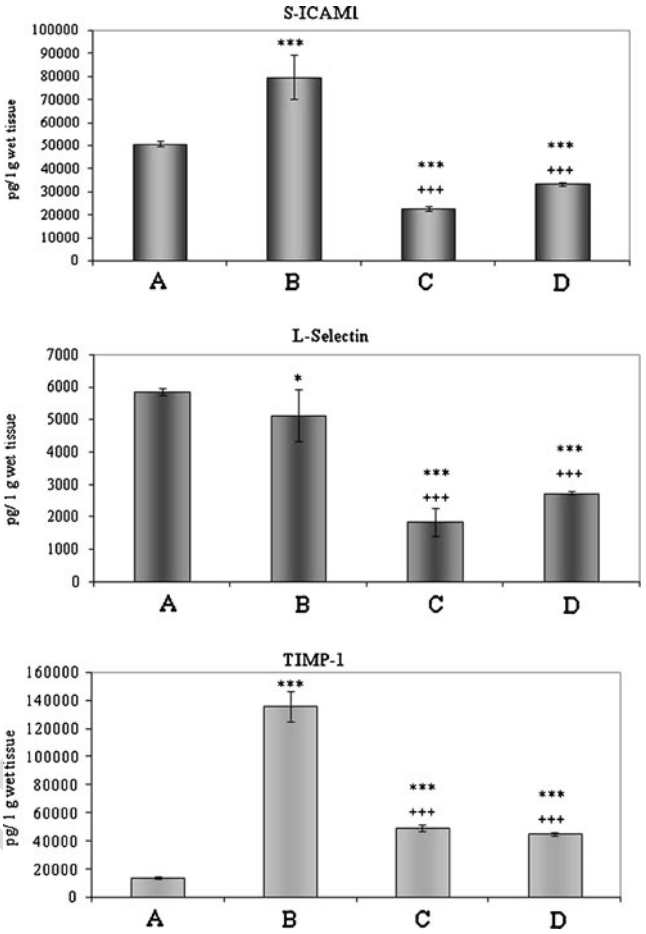

Fig. 4 Cytokine measurement by Luminex immunoassay showing the appearance on various cytokines in control intestine (*A*) and in small bowel tissue exposed to 6 h cold storage in UW (*B*) or 6 h cold preservation in PACAP-38-containing UW solution (*C*) and subsequent 3 h reperfusion period (*D*). Marked changes are observed in sICAM-1 (**a**), L-selectin (**b**), and TIMP-1 (**c**) expressions. Final values were given as picogram per gram wet tissue weight. Data are presented as mean ± SEM. $^{*}p<0.05$ vs. A, $^{***}p<0.001$ vs. A, $^{+++}p<0.001$ vs. B

Small bowel transplantation is increasingly performed in recent years, yet, clinically, there are still many obstacles to improve patient and graft survival. For most grafts, the preservation solution plays a fundamental role in minimizing the detrimental effects of ischemia during cold storage and subsequent reperfusion periods. The current clinical standard for small bowel consists of a vascular flush with cold UW solution. This solution has many advantages in the preservation of liver and kidney; however, for small bowel storage it still is unclear whether UW is optimal (Kokotilo et al. 2010; Roskott et al. 2011; Salehi et al. 2004). Several research efforts have been directed towards methods to protect against I/R injury, using modified composition of the commercially available solutions or adding new components appropriate for intestinal storage.

In the present study, we demonstrated that intestinal tissue PACAP-38 and PACAP-27 levels decreased in a time-dependent manner after 1 and 3 h cold preservation procedure. These changes were significant following 6 h cold storage. Our previous study showed similar tendency in the results of endogenous PACAP-38 concentration changes in warm I/R intestinal model (Ferencz et al. 2009). The reason for the decreased PACAP-38 levels after cold ischemia may be due to either excessive uptake by ischemic cells or decreased synthesis/increased degradation paralleling tissue degeneration. Similar observations have been made by others in an experimental ulcer model, where an acute decrease in PACAP immunoreactivity was observed (Mei and Sundler 1998). Values remained significantly higher in grafts stored in PACAP-38-containing UW solution. Interestingly, PACAP-38 levels increased above control values following 1 h preservation. Three and 6 h cold storage in PACAP-38-containing preservation solution resulted in significantly higher PACAP-38 and PACAP-27 levels in bowel tissue homogenates compared to only in UW-preserved grafts without PACAP-38. There are no data indicating the exact mechanism of the elevated values at the end of the reperfusion periods. It could be due to the decrease in intracellular cyclic adenosine monophosphate (cAMP) through the reduction of adenylate cyclase activity induced by hypoxia in endothelial cells in vitro (Yan et al. 1997). These changes were confirmed in an in vivo small intestine preservation study. Among these mechanisms, the cellular cAMP signal may represent a major determinant of the intestinal integrity after global ischemic preservation (Minor and Isselhard 1998). Studies confirmed that administration of PACAP-38 enhancing the cAMP level exerted tissue protection against I/R injury (Riera et al. 2001). Moreover, after extrinsic denervation, which is an indispensable procedure during intestinal transplantation, PACAP-38 concentration decreased in the stomach, but not in the small intestine. These findings suggested a dual intrinsic and extrinsic origin of the PACAP-containing nerve fibers in the small intestine (Hannibal et al. 1998). Another explanation of the present result is that PACAP-38 intake from the preservation solution and attached to the specific receptors could result in the anti-oxidant and protective effect to the bowel structure as described in our previous studies (Ferencz et al. 2009, 2010a, b).

I/R injury is one of the main factors affecting the function and structure of the small intestine, by generation of pro-inflammatory mediators including cytokines. The generated inflammatory cascade may activate leukocytes and endothelial cells, which ultimately lead to tissue inflammation, multiple organ dysfunction, and death. Following I/R in small bowel transplantation, the gut turns into a cytokine-producing organ, threatening graft and patient survival (Kostopanagiotou et al. 2011).

In the present study, we found that the expression of the sICAM-1 (CD54) and L-selectin (CD62L/LECAM-1) regulated upon activation was detectable in control bowel samples, and those after 6 h cold preservation in UW and subsequent reperfusion period. In contrast, 6 h cold storage in PACAP-38-containing UW solution caused strong reduction in the activation of these cytokines. Increased expression of sICAM-1 and L-selectin was also observed after renal I/R, and it was decreased in PACAP-treated groups in renal model (Horvath et al. 2010b). In fact, these adhesion molecules, involved in the distinct cellular crosstalk between leukocytes, platelets, T cells, and endothelial cells, can cause microvascular dysfunction and reperfusion damage (Vollmar and Menger 2011). The RANTES (CCL5) chemokine is not constitutively expressed; it is released during inflammation. In our model, the RANTES (CCL5) levels were increased in all groups, but slight reduction was observed in PACAP-treated groups. During inflammatory events, the transcription of matrix metalloproteinase-9 and its endogenous inhibitor TIMP-1 is induced by pro-inflammatory mediators. In our experiment, TIMP-1 showed a strong activation in 6 h preserved grafts without PACAP-38. PACAP-38-containing cold storage could decrease its activation. The anti-inflammatory actions of PACAP in several inflammatory models are partially mediated through its suppressing effect on cytokine/chemokine production (Delgado and Ganea 2001; Horvath et al. 2010b). In summary, our present results support the protective role of PACAP-38 in cold UW solution-stored and autotransplanted small intestine, which may have clinical relevance in bowel transplantation in the future.

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