Examination of PACAP expression and function in the inflammatory gastrointestinal diseases and in their models

Ph.D. thesis

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1. Introduction:

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide belonging to the secretin/glucagon/vasoactive intestinal peptide family. It was first identified in ovine hypothalamus based on the efficacy on influencing adenylate cyclase activity. PACAP has a widespread distribution in the body and exerts a wide range of physiological effects also in the gastrointestinal system. The occurrence and functions of PACAP and its receptors can be well demonstrated in neuroendocrine and interstitial cells, in the myenteric and submucosal plexus in the entire length of the gastrointestinal tract and also pancreas, gall bladder and liver. PACAP exerts a variety of functions in the intestine. PACAP influences motility of the intestinal wall, inhibits pacemaker activity of interstitial cells of Cajal, regulates sphincter function, affects on secretion of various glands and gastric acid. PACAP exerts relaxant activity in the body and fundus of the stomach and PACAP-immunoreactive fibers are often observed around and in the walls of blood vessels. The general vasodilatory action of PACAP is well established in several species and experimental models. This was also described for the stomach wall. Several in vivo and in vitro studies confirmed the general cytoprotective, antiapoptotic, antioxidant, and anti-inflammatory effects of PACAP. These effects are exerted mainly through G protein-coupled receptors, the specific PAC1 and the VPAC1/2 receptors, which are shared with VIP. In the intestinal system, PACAP has been shown to be protective in various models of intestinal injuries, such as ischemia-reperfusion, transplantation and inflammatory disorders. In small intestinal ischemia, both endogenous and exogenous PACAP have been shown to have protective effects. The anti-inflammatory activity of PACAP can be ascribed to inhibition of immune and inflammatory cells. PACAP decreases the release of inflammatory chemokines and cytokines such as TNF-α and IL-6, inhibits chemotaxis and phagocytosis. Hence, it is an important endogenous immunomodulatory peptide in many different models of inflammatory diseases. In humans, several studies have previously shown PACAP level changes in colon diseases. An earlier study found significantly lower levels of PACAP in sigmoid colon and rectum tumors compared to normal healthy tissue. Another study described significantly higher PACAP levels in patients with symptomatic diverticular disease. On the contrary, investigations of colon mucosa of children with ulcerative colitis found decreases in nerve fibers containing PACAP. In case of acute ileitis caused by experimental Toxoplasma gondii infection in mice, PACAP prophylaxis improved survival and anti-inflammatory cytokine response. A recent study has revealed direct antimicrobial effect of PACAP38 against Gram-positive and Gram-negative bacteria. All these results indicate that PACAP influences directly and indirectly the intestinal flora and bacterial colonization, which might be a link with increased susceptibility of PACAP deficient mice to intestinal inflammatory diseases and tumors. PACAP deficient mice have been shown to be more vulnerable to harmful stimuli to the central nervous system, but also peripheral organs including peripheral nerves, kidney, and the intestinal tract. For instance, applying the acute dextran sodium sulfate (DSS)-induced colitis model, PACAP deficient mice displayed 50% higher mortality rates as compared to wildtype mice. In addition, PACAP deficient mice were suffering from more severe chronic DSS-induced colitis, whereas

60% of diseased animals additionally developed colorectal tumors with aggressive phenotypes. Interestingly, naive PACAP deficient mice did not display histopathological changes in their intestinal mucosa. Hence, lack of endogeneous PACAP resulted in increased susceptibility of the murine host to intestinal inflammation and inflammation-induced colonic cancer development.

There is compelling evidence that the host microbiota constitutes a key factor in health and disease of vertebrates. In fact, it has been shown that the gut microbiota is essentially involved in a multitude of physiological processes including food digestion, fat metabolism, vitamin synthesis, intestinal angiogenesis, enteric nerve function, and protection from pathogens as well as immune cell development. Conversely, pertubations of this complex intestinal ecosystem termed dysbiosis are associated with increased susceptibility of the host to distinct intestinal (e.g., inflammatory bowel disease, irritable bowel syndrome, coeliac disease) as well as to extra-intestinal immunopathological conditions (e.g., multiple sclerosis, autism, depression, allergy, asthma, cardiovascular morbidities).

2.Aims:

1.Several in vivo and in vitro studies confirmed the general cytoprotective, antiapoptotic, antioxidant, and anti-inflammatory effects of PACAP. The aim of the present study was to investigate the effects of PACAP on intestinal epithelial cells having high turnover INT 407 cell line against different harmful stimuli, such as oxidative stress, in vitro hypoxia and gamma radiation.

2. Adherence of bacteria to the surface of intestinal epithelial cells is a crucial step in intestinal bacterial infections. Therefore, the aim of our study was to explore the role of PACAP in bacterial adhesion in small intestinal INT407 and colon adenocarcinoma Caco-2 cell lines. In addition, our studies were expanded to obtain further information on PACAP's effects on inflammatory processes in the intestinal system using INT 407 cell culture. It is well-known, that PACAP deficient mice suffer from more severe chronic DSS-induced colitis. In fact, it has been shown that the gut microbiota is essentially involved in a multitude of physiological processes, and dysbiosis is associated with several conditions such as inflammatory bowel disease. We therefore addressed whether PACAP deficiency was associated with qualitative and/or quantitative differences in the gut microbiota.

3. PACAP and its receptors can be well demonstrated in the entire length of the gastrointestinal tract. Several studies detect that PACAP exerts a variety of functions in the intestine. Our aim was to determine PACAP levels for some inflammatory diseases of the gastrointestinal tract using human samples.

3.Methods:

3.1. INT 407 cell culture

The INT 407 cell line isolated originally from human embryonic intestinal tissue was purchased from ATCC. INT 407 cells were cultured in Roswell Park Memorial Institute (RPMI 1640) medium (Lonza,Switzerland) supplemented with 10% fetal bovine serum (Biosera, USA) and 1% penicillin-streptomycin (Biosera, USA). Cells were passaged by trypsinization (Trypsin/EDTA; Biosera, USA), followed by dilution in RPMI medium containing 10% fetal bovine serum. Experiments started 24 h after incubation in humified 95% air and 5% CO_2 mixture at 37 °C in the medium.

3.2. Caco-2 cell culture

The Caco-2 cell line derived from human colon adenocarcinoma cell line was from ATCC. Caco-2 cells were cultured in DMEM high glucose/F-12 supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Biosera, USA). Cells were passaged by trypsinization (Trypsin/EDTA; Biosera, USA), followed by dilution in DMEM medium containing 10% fetal bovine serum. Experiments started 24 h after incubation in humified 95% air and 5% CO_2 mixture at 37 °C in the medium.

3.3. PACAP38

All of our studies were using PACAP38, which was synthetized by lab of Prof. Dr. Gábor Tóth in the Department of Medical Chemistry of the University of Szeged. The concentrations used for the experiments were determined in our earlier in vitro experiments.

3.4. Effect of PACAP on proliferation of INT 407 cells

3.4.1. Proliferation test

Effect of PACAP on cell proliferation was investigated using cells plated into 96-well microplates. Cells were randomly assigned to experimental groups: control cells with no treatment; cells treated with different concentrations of PACAP (10 nM, 100 nM, 1 μM). After 24 h of treatment, effect of PACAP on cell proliferation was examined by MTT assay (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, Hungary).

3.4.2. Clonogen assay

Effect of PACAP on clonogenic potential was examined by clonogenic survival assay. Cells were plated in six-well plates at 500 cells/ well. On the second day of experiment, cells were either left untreated or were treated with 100 nM PACAP. After 7 days of incubation, medium was removed and cell clones were washed and stained with Coomassie brilliant blue, and the colonies containing at least 50 cells were counted.

3.4.3.Oncology array

Investigating the effect of PACAP on expression of various cancer-related proteins Proteome Profiler Human XL Oncology Array was used. Cells were plated in six-well plates and were treated for 24 h according to the following experimental groups: control group of cells and cells treated with 100 nM PACAP. After incubation, supernatants were collected as described by the manufacturer. The procedure was performed as described by the manufacturer. Briefly, after blocking the membranes for 1 h, membranes were incubated with samples of supernatant at 2–8 °C overnight on a rocking platform. After washing, membranes were incubated in Detection Antibody Cocktail for 1 h. After washing steps, horseradish peroxidase-conjugated streptavidin was added for 30 min, then membranes were exposed to a chemiluminescent reagent. Array data were analyzed using ImageJ software.

3.5. Effect of PACAP on INT 407 cells exposed to gamma radiation

3.5.1.Radiation treatment

A telecobalt external irradiation equipment (Theratron 780C, average photon energy of 1.25 MeV) was applied for irradiation of the cells with a dose of 0.5, 1, 2, 3 or 4 Grays.

3.5.2. Cell viability

Cells were trypsinized and plated into 96-well microplates at 10000 cells/well. For detecting the effects of PACAP on cells exposed to irradiation, the following experimental cell groups were investigated: control group with no treatment; cells exposed to 100 nM PACAP; cell groups exposed to 0.5, 1, 2, or 4 Gy irradiation; cell groups treated with 100 nM PACAP 2 h before 0.5, 1, 2, 4 Gy irradiation, respectively. After 24 h, cell viability was assessed using MTT assay.

3.5.3. Clonogenic survival assay

Besides investigating cell survival, clone-forming ability of cells was also investigated by clonogenic survival assay. As described above, cells were plated in six-well plates at 500 cells/well. On the second day, cells in six wells were left untreated and cells of other six wells were treated with 100 nM PACAP 2 h before irradiation. After 7 days of incubation, the medium was removed and cell clones were washed and stained with Coomassie brilliant blue, and the colonies containing at least 50 cells were counted.

3.5.4. Phospho-ERK1/2 western blot

Cells seeded into six-well plates at the starting density of 10^6 cells/ well were assigned in the following experimental groups: control cells with no treatment; cells treated with 100 nM PACAP; cell groups exposed to 0.5 or 1 Gy irradiation; cells treated with 100 nM PACAP 2 h before 0.5 and 1 Gy irradiation, respectively. After 24 h, cells were harvested in ice cold lysis buffer containing the phosphatase-inhibitor Na3VO4 and protease inhibitor cocktail in phosphate buffered saline (Sigma, Hungary). After disruption, cells were centrifuged at 12,000 × *g* for 5 min at 4 °C. Protein samples were separated in 12% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and then transferred to nitrocellulose membrane. In the next

step, membranes were blocked with non-fat dry milk overnight then probed with anti-β-actin and anti-phospho-ERK1/2 (Cell Signaling Technology, MA, USA). Horseradish peroxidase-conjugated anti-rabbit IgG was used as secondary antibody (Bio-Rad, Hungary). Protein bands were visualized by the ECL chemiluminescence system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

3.6. Effect of PACAP against in vitro hypoxia

To investigate the effects of PACAP on cells under the influence of in vitro hypoxia, cell groups were examined as follows: control group of cells with no treatment; cells exposed to 100 nM PACAP alone simultaneously or 2 h before starting the $CoCl_2$-treatment; cells treated with 500 μM $CoCl_2$; cells either co-treated with 100 nM PACAP and 500 μM $CoCl_2$ for 24 h or pretreated with 100 nM PACAP for 2 h and then 500 μM $CoCl_2$ for 24 h. Cell viability was evaluated by MTT assay.

3.7. Effect of PACAP against oxidative stress

To investigate the effect against oxidative stress, cells were randomly assigned to one of the experimental groups: control group of cells (no treatment); cells exposed to 100 nM PACAP alone simultaneously or 2 h before starting H2O2-treatment; cells treated with 1 mM H2O2 for 24 h; cells either co-treated with 100 nM PACAP and 1 mM H2O2 for 24 h or pretreated with 100 nM PACAP for 2 h and then 1 mM H2O2 for 24 h.Cell survival was measured by MTT assay.

3.7.1. Small interfering RNA transfection

After studies with exogenously given PACAP, we performed experiments aiming to elucidate the role of endogenous PACAP by small interfering RNA transfection. The experiment was performed as follows: 5 nmol GAPD Control Pool, Non-targeting Pool and ADCYAP 1 siRNA (Dharmacon, GE Healthcare, Lafayette, CO, USA) dried pellets were dissolved in 1X RNase-free buffer. The composition of 1X RNase-free buffer is 60 mM KCl, 6 mM HEPES pH 7.5, and 0.2 mM MgCl2. All siRNAs were transfected into the cells using DharmaFECT1 transfection reagent (Dharmacon, GE Healthcare, Lafayette, CO, USA) in serum-free RPMI medium. Transfections were performed on human INT 407 cells cultured in 96-well tissue culture plates. Cells were seeded at 10^4 cells/well 24 h prior to transfection. Following the manufacturer's protocol, 25 nM GAPD Control Pool, Non-targeting Pool or ADCYAP1 siRNAs were dissolved in Dharma- FECT1 containing serum-free RPMI medium. 200 μl of siRNAs were added in the wells and samples were then incubated for 48 h. After transfection, cells were treated with different stimuli using PACAP and H2O2 for 24 h as follows: untreated control; cells exposed to 100 nM PACAP; cells treated with 1 mM H2O2 and cells stimulated with both 100 nM PACAP and 1 mM H2O2. The viability of cells was measured with MTT assay.

3.7.2. MTT assay

Following the incubation periods, cell viability was assessed by colorimetric MTT assay detecting the absorption of blue formazan dye particles produced by viable mitochondria. At the end of the incubation period, cells were washed twice with phosphate-buffered saline (PBS, Sigma, Hungary), then incubated with PBS-containing 0,5 mg/ml of MTT for 3 h at 37 °C in an atmosphere of 5% CO2. The solution was aspirated carefully and 200 µl dimethyl sulfoxide (Sigma, Hungary) was added to dissolve the blue-colored formazan particles and absorbance was measured by an ELISA reader (Dialab Kft., Hungary) at 570 nm representing the values in arbitrary unit (AU).

3.7.3. Statistical analysis

Each experiment was repeated minimum three times. Statistical analysis was performed by two-way analysis of variance. $p<0.05$ was considered as significant.

3.8. Effect of PACAP on bacterial adherence and gut microbiota

3.8.1. Determination of bacterial adherence

Before determination of bacterial adhesion, the living INT 407 and Caco-2 cells were counted by trypan blue and $3x10^5$ cells/well were plated into 24-well tissue culture plates. Both the small intestinal INT407 cells and the large intestinal Caco-2 cells were grown in their media supplemented with 10% fetal bovine serum without antibiotics in 5% CO_2 at 37°C. Half of the 24-well tissue culture plates contained cells, which were cultured in the above described medium supplemented with 400ng/ml PACAP. Next day the confluent monolayers were washed three times with 1ml Dulbecco's Phosphate-Buffered Saline (DPBS) before infection with $3x10^8$ bacterial cells in DPBS. After incubation for 3 h at 37°C in 5% CO_2 each well was washed three times with one ml of DPBS. To recover adherent bacterial cells, washed INT 407 and Caco-2 cells in each well were treated with 1 ml of 0.1% Triton-X100 and 0.25% trypsine in PBS for 10 min at room temperature. Each lysate was homogenized by repeated pipetting and 10 µl of their ten-fold serial dilutions were plated on Mueller-Hinton agar plates and incubated at 37°C for 24h. The following day, the colonies were counted to determine the number of bacteria that had adhered to the small and large intestinal cells. Adherent bacterial counts were obtained from three independent assays with each assay performed in triplicate wells. The investigated bacterial cells were *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 14028, *Klebsiella pneumoniae* ATCC 13833, *Enterococcus feacalis* (clinical isolate). Each experiment was repeated six times.

3.8.2. Cytokine array

Investigating the effect of PACAP on cytokine expression Proteome Profiler Human Cytokine Array kit (R&D Systems, Minneapolis, MN, USA) was performed. The investigated INT407 cells were plated in six-well plates and the following experimental groups were created: 1., control group of cells, 2., cells treated with 100 nM PACAP alone for 24 h, 3., cells exposed to 100ng/ml LPS for 24 h and 4., cells treated with 100 nM PACAP 2 h prior to 24 h-long 100 ng/ml LPS exposion. After incubation, supernatants were collected and were carried out according to the manufacturer's protocol. The kit contains all necessary contents. Briefly, after

blocking the membranes for 1 h and adding the reconstituted Detection Antibody Cocktail for another 1 h at room temperature, membranes were incubated with sample/antibody mixture at 2-8°C overnight. After washing, horseradish peroxidase-conjugated streptavidin was added for 30 min, then membranes were exposed to a chemiluminescent reagent. Array data were analyzed using ImageJ software. The experiment was repeated three times.

3.8.3. Statistical analysis

Statistical analysis was performed by two-way analysis of variance. $p<0.05$ was considered as significant.

3.9. Comparison of the gut microbiota composition in PACAP KO and wild type mice

3.9.1. Mice

PACAP gene-deficient mice and corresponding wild type (WT) counterparts were raised in open cages within an experimental semi-barrier under standard conditions (22–24 °C room temperature, 55 ± 15% humidity) in the animal facilities of the University of Pécs. Mice had unlimited access to autoclaved tap water and standard chow. Genotypes of mice were confirmed by polymerase chain reaction (PCR) as described elsewhere. All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments (BA02/2000-15024/2011).

3.9.2. Molecular analysis of fecal microbiota

Fresh fecal pellets were collected from corresponding mice at 2 weeks, 1 month, 3 months, 6 months, 12 months, and >15 months (i.e., between 15 and 18 months) of age, immediately snap-frozen in liquid nitrogen and stored at –80 °C until further processing. The examination was happened in the Department of Microbiology in Berlin Medical University. The main bacterial groups abundant in the murine intestinal microbiota including enterobacteria, enterococci, lactobacilli, bifidobacteria, *Bacteroides/Prevotella* spp., *Mouse Intestinal Bacteroides, Clostridium coccoides* group, and *Clostridium leptum* group as well as total eubacterial loads were assessed by quantitative real-time PCR (qRT-PCR). In this thesis we aside from the accurate and detailed description of the DNA analysis.

3.9.3. Statistical analysis

Medians and levels of significance were determined by Mann–Whitney *U* test (GraphPad Prism v5, La Jolla, CA, USA) as indicated. Two-sided probability (*p*) values of ≤ 0.05 were considered significant.

3.10. Measurement of PACAP level in human samples

We examined samples taken from patients suffering from ulcerative colitis (n=30) or Crohn's disease (n=23) and used samples obtained from patients having diarrhea without any morphological abnormality as controls (n=13), after obtaining patients' consent. In both groups of IBD 3 grades were distinguished: (1) chronic inflammation with lymphocyte and plasma cell infiltration without cryptitis or crypt abscess; (2) acute inflammation with cryptitis or crypt

abscess and neutrophil infiltration; (3) normal mucous membrane showing no signs of inflammation. For observing the effects of antibiotic therapy on PACAP level, colon samples of IBD patients treated with metronidazol or ciprofloxacin/metronidazol combination were also examined. We examined samples taken from patients suffering from antral gastritis (n=8) and ventricular ulcer (n=6). In case of antral gastritis, the data of the inflamed and normal (corpus) mucosa were compared, while in case of ulcer we compared the data of samples from the basis of ulcer, margin of ulcer and from the normal ventricular mucosa. In addition, PACAP levels of the samples were compared on the basis of *Helicobacter pylori* infection. The procedure used was in accordance with protocols approved by the ethical committee (no. 2784,3117, University of Pecs; 8-28/92 009-10 I 8EKU, ETT TUKEB, Ministry of Health, Hungary). Tissue samples were weighed and homogenized in ice-cold distilled water. The homogenate was centrifuged (12,000 rpm, 4°C, 30 min), and the supernatant was further processed for radioimmunoassay (RIA) analysis of PACAP38 and PACAP27 contents. For these measurements antiserum PACAP38 "88111-3" (working dilution, 1:10,000) and PACAP27 "88123" (dilution: 1:45,000) were used.

4. Results:

4.1. Investigation of the effect of PACAP alone on INT 407 cells

Effect of PACAP on cell proliferation was investigated with MTT assay. None of the applied concentrations led to significant changes in cell survival rate. For obtaining more precise results, clonogenic survival assay was performed. While MTT assay can only give information about a cell being alive or dead, clonogenic assay is for examining the outcome of severely damaged living cell not being able to replicate. We found that PACAP in the applied 100 nM concentration did not influence the number of cell colonies counted 7 days after. Besides investigating the effect of PACAP on cell viability and clone formation, its effects on expression of various cancer-related factors was also examined by Proteome Profiler Human XL Oncology Array Levels of carbonic anhydrase IX, Fox01 and p27 were significantly decreased in the cell culture supernatants after 24 h PACAP treatment. No changes could be detected in the expression of enolase 2 and galectin-3.

4.2. Investigation of the effect of PACAP on INT 407 cells exposed to gamma radiation

Viability of cells exposed to gamma radiation was measured using MTT assay. All applied radiation doses (0.5, 1, 2, 4 Gy) significantly altered the ratio of the living cells. PACAP treatment, added 2 h prior to irradiation, was not able to influence the altered survival rate.
In contrast to these findings, using clonogenic survival assay, PACAP was able to change the clonogenic potential of irradiated cells. Irradiation changed the clone-forming ability of irradiated cells depending on the used radiation dose: 0.5 Gy irradiation led to significant increase in clonogenic potential, whereas 2 Gy, 3 Gy and 4 Gy radiation doses resulted in lower proliferating activity. In all cases, PACAP pretreatment caused lower mitotic activity, which

was significant in cases of 0.5, 1, 2, Gy radiation doses. For examining the molecular biological background of these observations, expression of phosphorylated ERK1/2 was assessed by western blot. Our results showed that PACAP dramatically decreased the level of phosphorylated ERK1/2 in cells exposed to 1 Gy irradiation.

4.3. Investigation of the effect of PACAP against in vitro hypoxia

Viability of cells suffering from in vitro hypoxia was significantly decreased compared to control cells in both series of experiments. This decrease could not be either counteracted or enhanced by PACAP addition independently from the timing of PACAP treatment. PACAP treatment alone had no effect on cell survival.

4.4. Investigation of the effect of PACAP against oxidative stress

Cells exposed to oxidative stress evoked by 1 mM H2O2 showed lower survival rate. Pretreatment with PACAP could not change the ratio of living cells, whereas simultaneous treatment with PACAP was able to attenuate the survival-decreasing effect of oxidative stress significantly. After examining the effect of exogenously added PACAP, the role of endogenous PACAP was also investigated. For this purpose, ADCYAP1 small interfering RNA transfection was performed. After silencing, viability of cells was measured by MTT assay. Data of cells undergoing silencing were compared to those without posttranscriptional silencing. Hydrogen-peroxide treatment led to significant decrease in cell survival rate both in the siRNA treated cells and in non-silenced cells. Comparing the viability of cells in both groups, significant difference was detected between groups exposed to oxidative stress. The latter results may indicate a higher vulnerability of cells that underwent posttranscriptional PACAP silencing. When exogenous PACAP was added to H2O2-treated cells, the survival-decreasing effect of H2O2 was alleviated in both groups, while PACAP alone did not change the ratio of the living cells.

4.5. Effect of PACAP on bacterial adhesion

To investigate the effect of PACAP on bacterial adhesion PACAP-pretreated and non-pretreated INT407 and Caco-2 cell cultures were infected with the following bacteria: *Escherichia coli* ATCC 25922, *Salmonella* Typhimurium ATCC 14028, *Klebsiella pneumoniae* ATCC 13833 and *Enterococcus feacalis* (clinical isolate). In the current experiment, the influence of PACAP on the number of bacterial colonies adhered to small and large intestinal cells was investigated. PACAP pretreatment of INT407 cells was not able to influence the bacterial adherence. Furthermore, similarly to INT407 cells, infections of colon adenocarcinoma-derived Caco-2 cells could not be altered by PACAP-pretreatment.

4.6. Effect of PACAP on cytokine expression in INT407 cells

To elucidate whether PACAP has an effect on cytokine expression in INT407 cells, we used Human Cytokine Array. PACAP alone significantly elevated the expression of IL-8 and IL-18

changed slightly the expression of CXCL-1 (C-X-C motif ligand 1) and MIF (macrophage migration inhibitory factor). 100 ng/ml LPS exposure led to higher levels of CXCL-1, IL-8, IL-18 and MIF. These changes were significant in case of IL-8 and IL-18. PACAP-pretreatment was able to attenuate the LPS-induced elevated expression of IL-8 and CXCL-1. Both PACAP and LPS exerted a slight, but not significant, activating effect on MIF.

4.7. Comparison of the gut microbiota composition in PACAP KO and wild type mice

We collected fecal samples of age- and sex-matched mice starting 2 weeks postpartum until the age of 18 months. When assessing gene numbers of commensal enterobacteria including *Escherichia coli*, 2-week-old and 1-month-old WT mice harbored up to four orders of magnitude more enterobacteria in their intestines as compared to WT mice aged ≥3 months. Furthermore, PACAP deficient mice over 12 months of age exhibited less enterobacteria in their feces than 1-month-old and 3-month-old animals. Irrespective of the genotype, intestinal lactobacilli loads were approximately two orders of magnitude higher in ≤3-month-old mice than in older counterparts. Two-week-old WT mice harbored higher bifidobacteria gene numbers in their feces as compared to younger (except for 3-month-old) mice. Even though 6-month-old WT animals exhibited very low intestinal bifidobacteria numbers in their intestines. Remarkably, even in the youngest cohort, bifidobacteria were abundant only in very low numbers in the intestinal tract of PACAP deficient mice. When analyzing obligate anaerobic gram-negative commensals, fecal loads of *Bacteroides/Prevotella* spp. were highest in the youngest and lowest in the oldest cohort of WT mice. In PACAP deficient mice, however, fecal gene numbers of *Bacteroides/Prevotella* spp. slightly decreased until 3 months of age but were higher thereafter and comparable to loads observed in the youngest cohort. Fecal gene numbers of *Clostridium coccoides* were slightly lower in >15-month-old as compared to ≤3-month-old WT mice. In >6-month-old PACAP deficient mice, *C. coccoides* loads were up to 1.5 orders lower in the feces as compared to those detected in younger animals. When comparing total eubacterial gene numbers in fecal samples derived from WT and PACAP deficient mice at defined time points postpartum, no differences could be observed. Fecal enterobacteria loads, however, were two orders of magnitude lower in 1 month old PACAP deficient as compared to age-matched WT mice. PACAP deficient mice harbored more enterococci in their intestines as compared to WT counterparts, whereas the opposite was true for ≥6-month-old mice. Obligate anaerobic gram-negative commensals such as *Bacteroides/Prevotella* spp. were up to 1.5 order of magnitude higher in ≥12-month-old PACAP deficient as compared to age-matched WT control animals, which also held true for *Mouse Intestinal Bacteroides* in respective 1-month-old and 6-month-old mice. Whereas lactobacilli were higher in fecal samples derived from PACAP deficient as compared to WT mice of the respective oldest cohorts. PACAP deficiency is accompanied by distinct changes in fecal microbiota composition with virtually absent bifidobacteria as a major hallmark that might be linked to increased susceptibility to disease.

4.8. Measurement of PACAP level in human samples

We found that both PACAP38 and PACAP27 levels of normal mucous membrane samples of UC patients were much lower than in the inflamed samples, but there were no difference in case of CD patients. Measuring PACAP levels, significant elevation of both PACAPs could be detected in samples obtained from colon of UC patients showing acute inflammation. Studying the possible effect of antibiotics on PACAP levels in IBD patients revealed a significant decrease of both PACAP38 and PACAP27 level in UC patients receiving antibiotics treatment. In samples from patients suffering from antral gastritis we did not observe significant difference in the inflamed antrum and in the intact corpus samples PACAP38-levels. We did not find any significant difference between the *Helicobacter pylori* positive and negative samples. In case of gastric ulcer we did not observe significant difference among the PACAP38 levels of samples from the basis of ulcer, margin of ulcer and from the normal ventricular mucosa.

5. Discussion:

5.1. Effects of PACAP on intestinal epithelial cells against different harmful stimuli

The aim of our study was to investigate the effect of PACAP in human intestinal epithelial cells. We tested whether PACAP has influence on cell proliferation and clone-forming ability. Furthermore, effect of PACAP on expression of various cancer-related factors was also investigated using Proteome Profiler Human XL Oncology Array. We found that PACAP treatment did not change the number of viable cells measured by MTT assay. Besides MTT assay, clonogenic assay was also performed in order to investigate whether PACAP influences the clonogenic potential of cells. PACAP did not show any effect on cell proliferation and clonogenic potential. It is in accordance with previous observations where PACAP alone did not change the ratio of living cells if added in vitro. Moreover, data obtained from Human XL Oncology Array showed significant decrease in the expression of levels of carbonic anhydrase IX, FoxO1 and p27. Expression level of carbonic anhydrase IX, related to renal malignancy was significantly lower in PACAP-treated samples. FoxO1 having effect on apoptosis and cell cycle was also significantly reduced by PACAP. Furthermore, decrease of expression level of p27/Kip1 could also be detected. p27 belongs to cyclin-dependent kinase inhibitors and exerts both positive and negative regulation of cyclin-dependent kinase activity. These data might indicate the cell cycle-regulating effect of PACAP in INT 407 cells, but no major influence under normal circumstances. In the second set of experiments, effects of PACAP against different stressors were investigated. First we tested the effect of PACAP in INT 407 cells exposed to $CoCl_2$ evoking in vitro hypoxia. Neither pretreatment nor co-treatment with PACAP could successfully influence cell survival. Furthermore, effect of PACAP against gamma radiation was examined. The damage of small intestinal epithelial cells in patients receiving radiotherapy is a major side effect. Our aim was to investigate whether PACAP could attenuate the damage of small intestinal epithelial cells characterized by high turnover. We found no effect of PACAP on viability of irradiated cells similar to in vitro hypoxia, while it caused decrease in clonogenic potential of cells exposed to gamma radiation. For examining the molecular biological background of these observations, expression of phosphorylated ERK1/2

was assessed by western blot. Our results showed that PACAP dramatically decreased the level of phosphorylated ERK1/2 in cells exposed to 1 Gy irradiation. ERK is a member of an anti-apoptotic signal transduction pathway, so our investigate support that PACAP is increased the radiation cell damaging effects. In case of another applied stressor, PACAP could counteract the survival-decreasing effect of oxidative stress, but only when it was added simultaneously with H_2O_2-treatment. The mechanism, by which ionizing radiation leads to cellular damage, is similar to events evoked by oxidative stress, where hydrogen peroxide treatment results in formation of reactive oxygen species leading to cellular damage. Gamma radiation rapidly generates reactive oxygen species (ROS), hydroxyl radical (OH), ionized water (H2O+), hydrogen radical (H) and hydrated electrons (eaq−), then secondary ROS products are formed (O2 − and H2O2). Formation of these ROS leads to a cascade of events, which, together with the reactive nitrogen species result in cellular damage. Investigating the effect of PACAP against gamma irradiation, we found that it did not influence cell viability, when we added PACAP 2 h prior to irradiation. Cell viability was decreased both by hydrogen peroxide treatment and gamma radiation, but PACAP was able to increase the cell survival only in case of H_2O_2 treatment, when PACAP was added simultaneously. In contrast, PACAP pretreatment could not counteract the toxic effect of oxidative stress. Besides investigating the ratio of living cells using MTT assay, clonogenic assay was also performed. Clonogenic assay is a more sensitive procedure providing information on the severity of cell damage if a cell is alive. It can give accurate information about the cell's ability of being replicated. In this experiment we found that PACAP decreased the clonogenic potential of irradiated cells in cases of all examined radiation doses, while PACAP treatment without irradiation did not worsen the clone-forming ability of INT 407 cells. Detection of clonogenic potential of cells exposed to H_2O_2-induced oxidative stress was not performable because H_2O_2-treated cells are not able to survive the long-lasting procedure of clonogenic assay (data not shown). These results suggest that effects of PACAP depend on the time-point of the application. Previously, PACAP has been shown to exert protective effect in HTR-8/SVneo cells when it was added 2 h prior oxidative stress, while PACAP co-treatment could not counteract the effect of H_2O_2. These data, together with our current ones, suggest that effects of exogenous PACAP depend not only on the exact cell type but on the time-point of application and the applied stressor. In the last set of our experiments, the role of endogenously present PACAP was examined using posttranscriptional RNA silencing. Our examined cell type isolated from embryonic tissues could contain endogenous PACAP in cells developing in direction of enteroendocrine cells. Presence of endogenous PACAP in small intestinal epithelial cells is supported by immunohistochemical observations. Our data showed that cells undergoing ADCYAP1 siRNA transfection responded with higher sensitivity to H2O2-induced oxidative stress. It is in accordance with previous observations, which demonstrate higher vulnerability of tissues of PACAP deficient mice compared with those of wild type mice. Studies performed on primary kidney cell cultures obtained from PACAP deficient mice also revealed susceptibility to oxidative stress. However, no significant difference between wild type and knockout cells could be detected under normal conditions. Other studies described similar relations in vitro, for example, earlier study found higher vulnerability of cerebellar granule cells against cellular stressors. This higher sensitivity observed only in case of exposition to different stressors, could also be detected in vivo in case of renal ischemia/reperfusion, small bowel ischemia and large intestine inflammation. These

data, in accordance with our current results suggest the protective role of endogenous PACAP against different harmful stimuli in different tissues under different circumstances. Taken together, our current findings indicate that both exogenous and endogenous PACAP plays a regulatory role in small intestinal cells, but this is restricted to certain conditions, such as type of stressor and timing of application.
Comparing the results of experiments using 3 different stressors (oxidative stress, hypoxia, ionizing radiation) we can conclude that PACAP is able to protect cells against oxidative stress both exogenously and endogenously but it is not able to enhance the survival of INT 407 cells exposed to gamma radiation or in vitro hypoxia.

5.2. Effect of PACAP on bacterial adhesion

In the present study we found that PACAP could alter the cytokine expression of INT407 small intestinal cells alone and especially after LPS exposure, indicating that the decreased cytokine levels after endotoxin insult can be an additional factor in its anti-inflammatory effect in several intestinal inflammatory conditions. However, we did not find any direct effect on bacterial adhesion, suggesting that PACAP does not affect bacterial adhesion on intestinal cells directly, but is rather involved in the inflammatory reactions induced by different pathogens. Our finding that PACAP did not directly influence bacterial adhesion is of importance in the light of previous findings indicating that PACAP might have direct effects on bacteria and other pathogens. A recent study has proven that PACAP and its related peptides and analogs are able to exert direct antibacterial effects. Both PACAP38 and 27, as well as related peptides, VIP and secretin, had antibacterial effects against Gram-negative bacteria, such as Escherichia coli. PACAP could act against the Gram positive Staphylococcus aureus. Another assay showed that PACAP had moderate sterilizing effect against Pseudomonas aeruginosa and Escherichia coli, an effect less pronounced by the other peptides. PACAP even had a moderate activity against Bacillus cereus. The mechanism of this effect was found to be a membrane permeabilization effect, without causing toxic side effects, as shown by the undisturbed hemolytic activity on red blood cells. All these data point to the possibility of PACAP acting directly on bacteria. We hypothesized that PACAP might also influence the adhesion of bacteria to the intestinal wall, but we found no effect in the adhesion assay. Therefore, based on our currect knowledge, it seems that PACAP can exert a protective effect on bowel inflammatory conditions via both direct antibacterial as well as cytoprotective actions, without influencing the adhesion of bacterial to the intestinal wall. As a next step, therefore, we investigated the effects of PACAP on cytokine expression of INT407 cells. As PACAP is a known modulator of inflammatory cytokine and chemokine production in various cells, we aimed at testing this effect in small intestinal cells. We found that PACAP altered the expression of several cytokines. IL-8 is a member of the chemokine family identified as a strong chemotactic factor. Interleukin-8 plays a crucial role in inflammatory, autoimmune and infectious diseases. In our present study, we detected signicantly elevated expressions of IL-8 upon exposure to LPS. PACAP was able to counteract the induction of IL-8 expression. Our finding is in accordance with those of Zhang et al., who found expression-decreasing effect of PACAP in ARPE cells stimulated with IL-1β. Besides IL-8, we found significantly elevated expression of IL-18 in LPS-induced samples, but in this case no effect of PACAP on it could be observed. Moreover, increase in expression of

CXCL-1 could be measured upon LPS exposure. PACAP-pretreatment behaved in an opposite way, it was able to significantly decrease the activation of CXCL-1. Delgado and colleagues have previously decribed expression-decreasing effect of PACAP in case of LPS-stimulated peritoneal macrophages and microglial cells. In summary, PACAP is able to alter the expression of several cytokines. This has been demonstrated in many different cell and tissue types, such as lymphocytes, astrocytes and microglial cells, in the retina in a chronic hypoperfusion model and in the kidney, in diabetic and ischemic nephropathy. The effects of PACAP on the cytokine expression varies between cells and also depends on the type of injury. In many cases, PACAP alone does not affect cytokine expression, but can counteract the injury-induced alterations. Our observations indicate that while PACAP has no direct action on the bacterial adhesion to the intestinal wall, it can counteract the endotoxin-induced effects on cytokine expression, possibly contributing to the well-known intestinal protective effects of the peptide.

5.3. Comparison of the gut microbiota composition in PACAP KO and wild type mice

The microbial colonization of the intestinal tract starts immediately after birth with facultative anaerobic commensal species such as enterobacteria, enterococci, and lactobacilli as the first colonizers. Irrespective of the genotype, fecal gene numbers of respective commensals declined with progressive aging, particularly after 3 months of age. Conversely, obligate anaerobic bacterial species including *Bacteroides* and *Clostridium* species, but also bifidobacteria, usually establish gradually in mice and men. Our results are well in line, given that *Mouse Intestinal Bacteroides* increased in either mice after 1 month of age, for instance, whereas *Bacteroides/Prevotella* spp. were lowest in feces of >15-month-old wild type but not PACAP deficient mice. Strikingly, bifidobacteria were virtually absent in the intestinal tract of PACAP deficient mice, even in still breastfed 2-week-old infants. In WT mice, however, bifidobacterial loads were highest in 2-week-old mice and subsequently declined after 3 months of age. Whereas bifidobacteria are considered important for intestinal homeostasis, a delayed bifidobacterial colonization of the intestinal tract during infancy results in increased susceptibility to diseases in childhood and later in life. For instance, dysbiosis with decreased bifidobacterial numbers have been associated with distinct immunopathologies such as inflammatory bowel disease, irritable bowel disease, celiac disease, and atopic disease. Genome-based analyses revealed a number of factors that might explain host-beneficial mechanisms exerted by bifidobacteria: production of lactic acid and acetic acid, thereby lowering the intraluminal pH, exerting functional oxidative stress response mechanisms, and thus supporting the host phalanx angainst invading (entero) pathogens as well as providing metabolic features such as production of vitamins, hydrolysis of bile salts, production of conjugated linoleic acid, and degradation of oxalate. Importantly, a very recent report provided a direct link between bifidobacteria and neuromodulatory activities, given that a particular *Bifidobacterium dentium* strain was able to modulate visceral sensitivity in the intestinal tract by production of γ-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the central nervous system of mammals. It is, hence, highly likely that the lack of intestinal bifidobacteria observed in PACAP deficient mice might be one key factor explaining their

vulnerability to cell-toxic stimuli and increased susceptibility to immunopathological conditions in both intestinal and extra-intestinal compartments.

5.4. Measurement of PACAP level in human samples

PACAP may has protective effect on inflammatory bowel disease. In colon mucosal axons of dogs suffering from IBD increased experession of PACAP is observed. The accurate patomechanisms of IBD are unknown, but the role of oxydative stress, and reactive oxygen metabolits in the pathogenezis of disorders are well-known. Our earlier studies show, that PACAP is able to protect cells against oxidative stress. Because of our goal was to determine PACAP levels in inflammatory bowel disease using human samples, we compared the inflammatory samples with samples obtained from patients having diarrhea without any morphological abnormality as controls. We found that both PACAP38 and PACAP27 levels in patients suffering from Crohn disease was similar comparing the data of normal and inflammed samples both in acut and in chronic group. Measuring PACAP levels, significant elevation of both PACAPs could be detected in samples obtained from colon of UC patients showing acute inflammation. We can explain the high PACAP levels with the PACAP anti-inflammatory effect. The quantity of PACAP was elevated by the effect of present inflammatory citokins in the tissues. Studying the possible effect of antibiotics on PACAP levels in IBD patients revealed a significant decrease of both PACAP38 and PACAP27 level in UC patients receiving antibiotics treatment. The intestinal flora plays an important role in the pathogenesis of inflammatory bowel disease and in the development of complications. Changes of intestinal flora, especially decreased diversity in the intestinal flora is related to the chronic inflammatory bowel disease. Certain pathogens' like virulent *Escherichia coli* strains, certain *Bacteroides* species, and the *Mycobacterium avium* role has previously been associated with pathogenesis of the inflammatory bowel disease. Antibiotics may reduce the concentration of these bacteria in the lumen of the intestine and alter the gut flora's composition. In clinical practice, mainly ciprofloxacin and metronidazole are used for inflammatory bowel disease. In our study the patients were treated with metronidazol or ciprofloxacin/metronidazol combination, this presumably reduced the inflammatory and immune cells in the tissues, in this context decreased the amount of PACAP. Besides the small and large bowel, the PACAP also affects the function of the stomach. We examined samples taken from patients suffering from antral gastritis (n=8) and ventricular ulcer (n=6). In samples from patients suffering from antral gastritis we did not observe significant difference in the inflamed antrum and the intact corpus samples PACAP38-levels. We did not find any significant difference between the *Helicobacter pylori* positive and negative biopsy samples. In the case of gastric ulcer we observed difference in the PACAP38 levels of samples from the margin of ulcer and from the normal ventricular mucosa. Thus, our results was similar like in rats in a previous experimental study, that in the samples from the margin of ulcer reduced the PACAP expression. However, in our study analyzing the values, the difference was not significant. Presumably, the difference is due to animal studies contain controlled, other diseases-free, completely homogenous groups, however in human studies the patients are more diverse, than the induced ulcer models in rats.

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