INVESTIGATION OF TRANSIENT RECEPTOR ANKYRIN 1, VANILLOID 1 ION CHANNELS AND PITUITARY ADENYLATE CYCLASE POLYPEPTIDE IN THE UTERUS AND MAMMARY GLAND

PhD Thesis

Krisztina Pohóczky

Pharmacology and Pharmaceutical Sciences Doctoral School

Neuropharmacology Program

Head of the Doctoral School and Program Leader: Erika Pintér MD, PhD, DSc

Supervisors: Zsuzsanna Helyes MD, PhD, DSc and Levente Czeglédi PhD

University of Pécs, Medical School

Department of Pharmacology and Pharmacotherapy

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INTRODUCTION

1. Transient Receptor Potential (TRP) ion channels

The Transient Receptor Potential Ankyrin 1 (TRPA1) and Vanilloid 1 (TRPV1) are non-selective cation channels predominantly localized on capsaicin-sensitive peptidergic sensory neurons and mediate pain and inflammation [1]. TRPV1 or „capsaicin receptor" is activated by noxious heat (>43°C), protons (pH>5.5), bradykinin, lipoxygenase products and anandamide produced during inflammation and tissue injury [2]. TRPV1 is also expressed in the central nervous system (CNS) and on several non-neuronal cells in the skin, kidney, lung, testis, pancreas, spleen, cornea, and the uterus [3], [4]. TRPA1 is also activated by various chemical and physical stimuli, such as noxious cold (<17C°), allyl-isothiocyanate, cinnamaldehyde, as well as endogenous ligands like hydrogen peroxide, formaldehyde, methylglyoxal and acrolein produced during inflammation and tissue damage [5]. The pathophysiological relevance of TRPA1 has been shown in inflammatory diseases of the respiratory, cardiovascular and gastrointestinal tracts. Similarly to TRPV1, functional TRPA1 was also described on enterochromaffin cells, synoviocytes, fibroblasts, melanocytes, pancreatic beta cells, epidermal keratinocytes, intestinal epithelial cells, and macrophages, as well as human endometrium cells besides sensory neurons [6]. Although the physiological/pathophysiological relevance of non-neuronal TRP is unknown, a cross-talk has been proposed between non-neuronal and neuronal TRP channels [4], [7]. The expression of TRPV1 at protein level has been shown in the intact human endometrium at both neuronal and non-neuronal sites [4], [8]. Although the non-neuronal receptor expression was steady during the menstrual cycle, neuronal TRPV1 expression presumably has an estrogen-dependent regulation The consistent upregulation of TRPV1 in the peritoneal and endometrial tissues of women with chronic pelvic pain suggests its potential significance in various gynecological pain symptoms [8]. Further research revealed increased TRPV1 expression at both neuronal and non-neuronal sites in the peritoneal endometriosis lesions and endometrioma [3]. Despite these data on TRPV1 expression in the human endometrium and association with constant severe pelvic pain, there are no data about its expression in DIE. Furthermore, there is no information about TRPA1 expression in the human endometrium at all.

2. Macrophage migration inhibitory factor (MIF)

MIF was originally identified as a T-cell derived lymphokine [9], it regulates immune responses and pain, but its specific receptor has not yet been identified. It is constitutively expressed by macrophages and T-cells, neurons, vascular endothelial cells and smooth muscle cells [10]. A hormonal cycle-dependent expression of MIF in was described in the human endometrium, which was elevated during the late proliferative and early secretory phases predominantly driven by estrogen [11]. MIF is also involved in human ectopic endometrium cell proliferation, and has been proposed a biomarker of endometriosis.

3. Pituitary adenylate cyclase activating polypeptide (PACAP)

PACAP is a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family and was first isolated from ovine hypothalamic extracts [12]. Although PACAP has poor metabolic stability and short plasma elimination half-life, it exerts important biological activities [13], [14]. Since its discovery, PACAP has been described in peripheral organs throughout the body [15]–[17] and its effects are mediated by specific receptors on the target cells: the PACAP preferring PAC1 receptor and two VIP-shared VPAC1 and VPAC2 receptors. Brain-derived neurotrophic factor (BDNF) is a member of neurotrophin family described in the development and survival of neuronal cells. A link between PACAP and BDNF has been described in the central nervous system (CNS) [18]. A recent article reports the presence and lactation-dependent alterations of BDNF in the sheep mammary gland [19]. The author suggests that the presence of BDNF acts as paracrine/autocrine signal responsible for a crosstalk between hormonal stimulation and the local micro environment.

AIMS

Our primary aims were the following:

1) To investigate the estrogen-dependent regulation of TRPA1 and TRPV1 receptor proteins in the rat endometrium

Although TRPV1 and TRPA1 have been shown in human eutopic and ectopic endometrium, their regulation and functional importance are not known. Furthermore, there are no data regarding the link between estrogen receptor activation and the expression of TRP channels and MIF in the normal endometrium related to different hormone levels. Animal models are important to precisely investigate their alterations during sexual maturation, the influence of

estrogen/gestagen actions, and estrogen receptor-dependent mechanisms. Therefore, we aimed to describe the expression of TRPV1 and TRPA1 in the rat endometrium at mRNA and protein levels, as well as their hormone-dependent changes in correlation with MIF.

2) To examine the local expression of the TRPA1 and TRPV1 ion channels in rectosigmoid deep infiltrating endometriosis (DIE)

Despite these data on TRPV1 expression in the human endometrium and association with constant severe pelvic pain, there are no data about its expression in DIE. Furthermore, there is no information about TRPA1 expression in the human endometrium at all.

Therefore, our goal was to describe the expression of TRPV1 and TRPA1 receptor at mRNA and protein levels in rectosigmoid DIE lesions in comparison with the eutopic and intact human endometrium, as well as to find potential correlations with the clinical symptoms.

3) To study the PACAP in the sheep mammary gland and milk, and in the lamb plasma after suckling

a) in the plasma of lambs in a time-dependent manner after suckling; *b)* in the plasma related to age; *c)* in the milk in comparison with the respective plasma and during the daily cycle. Furthermore, we also examined the expression of PACAP, VIP, their receptors and BDNF mRNA in the mammary gland.

MATERIALS AND METHODS

1. Samples

1.1. Rat samples and treatments

Four-week-old and four-month-old female Wistar rats were implanted with s.c. bee wax pellets containing the synthetic estrogen analogue DES (100 mg, w2 mg daily release), or were administered daily 2 mg s.c. progesterone (Sigma–Aldrich) dissolved in sesame oil for three consecutive days (n=5/group) [20]. When given in combination, progesterone administration started 2 days after DES pellet implantation. In a separate group of mature rats (n=5), ovariectomy was performed in order to investigate the effect of reduced endogenous hormone production. Samples were taken 10 days after completing the treatments. All experiments were approved by the Ethics Committee on Animal Research of Pécs University according to the Ethical Codex of Animal Experiments (licence: BA02/2000-11/2011).

1.2. Study participants and tissue

Twenty-seven women, aged between 18 and 45 years, underwent laparoscopic surgery due to chronic DM or subfertility with no history of pain and were grouped as follows: Group 1 (n=15), severe DM was found in conjunction with rectosigmoid DIE. Group 2 served as controls, patients with uterine fibroid-induced moderate DM (n=7), and Group 3 created from patients with tubal infertility with no pain (n=6). Autologous eutopic endometrium (n=6), ectopic endometrium from rectosigmoid DIE nodules (n=15) and healthy rectosigmoid bowel wall samples (n=15) from intact resection margins were matched with endometrial samples of women diagnosed with uterine fibroids (n=7), marked as negative controls. Endometrium of patients with tubal infertility but with no detectable gynecological pathology at laparoscopic inspection and no history of pain or endometriosis were evaluated as control samples (n=6). The research project was approved by the institutional ethics committee of University of Pécs Medical School, Hungary with a registration number of 5816.

1.3. Sheep samples

Samples were collected from Hungarian Tsigai sheep according to a protocol approved by the Institutional Ethic Committee (license number: 28/2012 DE MAB).

Plasma samples were collected under calm circumstances in a standardized manner, causing minimal distress for the animals. Plasma samples were collected into ice-cold evacuated plastic plasma collection tubes containing the anticoagulant EDTA (Sigma Aldrich, St Louis MO USA) and the peptidase inhibitor aprotinin (Sigma Aldrich, St Louis MO USA). For the suckling-related measurements, 10 mL plasma samples were taken from the jugular vein by the same method from 3-month-old suckling lambs (n=9) before (0 h) and 1 and 2 h after suckling (1 h, 2 h). In addition, samples were also taken from the jugular and mammary veins of their 3-year-old mothers (n=8), representing systemic venous plasma and that directly from the mammary gland vein, respectively.

Milk samples (5 mL/ animal) of lactating ewes were harvested in the morning into ice-cold centrifuge tubes. To evaluate daily changes of PACAP38-like immunoreactivity (PACAP38-LI) in milk whey, we collected milk samples from 4 lactating ewes (5 mL/ animal), at 6 different time points (04:00, 08:00, 12:00, 16:00, 20:00 and 24:00).

Animals were slaughtered following a conventional procedure according to EU regulations (Council Directive 93/119/EC of 22 December 1993 on the protection of animals at the time of slaughter). 10 mL blood, 1 cm^3 mammary gland (from the glandular component of the

mammary gland) and uterus samples (as positive controls for VIP mRNA expression) were harvested from 3-month-old (± 2 weeks), 3-year-old (± 4 months) and 10-year-old (± 1 year) animals (n = 4/ group) immediately after slaughtering.

2. Methods

2.1. Primary cultures of rat endometrial cells and *in vitro* treatments

Primary endometrial cell cultures were prepared in order to test the functionality of TRPV1 and TRPA1 receptors by detecting agonist-evoked Ca^{2+} influx responses, as well as to determine their ER activation-induced expression regulations and correlation with the MIF. Cells were pre-incubated for 18 h with the physiological concentration of 200 pM 17β-estradiol (Herbison 2009) having relative selectivity to ERα, and the same concentration of the non-selective ERα and ERβ receptor agonist DES was used in our in vivo experiment [21]. To evaluate the effects of chronic DES pretreatment in *our in vitro* cell culture, test the functionality of the TRP receptors, and find a direct link between the MIF cytokine and TRPA1/TRPV1, we made primary endometrial cell cultures from DES-pretreated (100 mg, w/w2 mg daily release) and placebo-pretreated 4-month-old rats for 10 days (bee wax pellet without active ingredient). Sub-confluent phase of the cultured endometrial cells was treated for 24 h with 1 mg MIF/ml, 330 nM capsaicin and 3.3 mM formalin respectively and the relative gene expression ratios of Trpa1, Trpv1, iNOS and Il1b were measured by qPCR.

2.2. RNA extraction and quantitative real-time PCR

In general, total RNA was extracted using TRI Reagent (Molecular Research Centre, Inc., Cincinnati, OH, USA) and the Direct-Zol RNA isolation kit, and then treated with DNase I (both supplied by Zymo Research, Irvine, CA, USA) to remove genomic DNA. RNA was measured with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA).

The method of the first-strand cDNA synthesis is based on the sample type: for rat uterus sapmles we used the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) with oligo(dT)18 primers. Human and sheep RNA samples were reverse transcribed with MaximaTM First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Amplifications were carried out under the following conditions: 95°C (10 min), followed by 40 cycles of 95°C (15 s), 60 8C (45 s) and 72 8C (45 s) in all cases. Reactions were carried out in triplicate; relative expression ratios were calculated using MxPro QPCR Software

(Agilent Technologies) with DDCt method. Primer efficiencies were taken into account when calculating gene expression ratios [22].

2.3. Immunohistochemistry

Rat uterus sections were treated with rabbit polyclonal anti-TRPV1 (Neuromics, Edina, MN, USA), anti-TRPA1 (Abcam, Cambridge, UK) and anti-MIF (Life Technologies) antibodies at a 1:1000 dilution. In the case of human endometriosis samples, we used rabbit polyclonal antibody against the TRPA1 (ab68847, Abcam, Cambridge, UK) and guinea pig polyclonal antibody against the TRPV1 receptor (GP14100, Neuromics, Edina, MN,) diluted to 1:300 and 1:100, respectively. After appropriate washing, slides were further incubated with the EnVision system anti-rabbit secondary antibody conjugated with horseradish peroxidase (DakoCytomation, Carpinteria, CA, USA) or VECTASTAIN ABC-Peroxidase Kit- Guinea Pig IgG (PK-4007, BioMarker Ltd., Budapest, Hungary) for 30 min at room temperature. Standardization was made using routinely performed positive and negative controls for each staining parameter. Negative control slides were reached incubating normal endometrium with tris-buffered saline instead of primary antibody. Immunopositivities were evaluated by a semi-quantitative scoring system ranging from 0 to 3 (0, no staining; 1, weak staining in the majority of cells with only focal moderate staining; 2, moderate staining in the majority of cells with only focal strong staining; 3, strong staining in the majority of cells). Human endometriosis slides were scanned using an automatic digital slide scanner (Pannoramic Midi II, 3DHistech, Hungary) yielding high-quality digital images of the entire samples.

2.4. Ratiometric technique of [Ca2C]i measurement

Sub-confluent phase of the cultured endometrial cells was determined with phase-contrast microscopy on day 7. Cultures were stained with the fluorescent Ca^{2+} indicator dye fura-2-AM, as described in detail previously [23]. [Ca2C]i measurement was done in extracellular solution (ECS) by fluorescence microscopy: ECS and the test solutions were given via separate tubes and rapid solution changes were controlled by the fast-step perfusion system. Fluorescence images were taken after illuminating the cells alternately at 340 and 380 nm light generated by a monochromator. The emitted light (510 nm) was measured with Axon Imaging Workbench 2.1 (AIW) software. The fluorescence ratio F340/F380 was monitored (rate 1 Hz) continuously for up to 5 min and R values were generated by the AIW software. Baseline fluorescence was read from the period of recordings taken before exposing the cells to the TRPV1 agonist capsaicin (330 nM) or the TRPA1 agonist formalin (3.3 mM).

2.5. RIA measurements

PACAP38-LI in the plasma, milk and mammary gland was determined with sensitive PACAP38 specific RIA technique developed in our laboratory and concentrations of the peptide were calculated with the help of a calibration curve [24], [25]. The antiserum used in the RIA is C-terminal specific, without affinity for other members of the VIP/secretin/glucagon peptide family. Synthetic peptides were used as RIA standard ranging from 8 to 2 000 fmol/mL. For the RIA measurments, we used 2 mL plasma sample, 10 mL milk sample and 280 mg mammary gland sample. After 48 h incubation at 4 °C, the antibody-bound peptide was separated from the free one by addition separating solution [content described previously by Jakab et al. [25]. Samples were centrifuged and then gently decanted and the radioactivity was measured by a gamma counter (Gamma, type: NZ310).

2.4. Enzyme-linked immunoassay (ELISA)

We measured VIP-LI in the same plasma and milk samples as described for PACAP38-LI using a specific ELISA Kit (Sincere™, Sincere Biotech Co., Ltd, Beijing, China) according to the manufacturer's protocol that is proven to be specific for ovine VIP. The reaction was evaluated at absorbance of 450 nm using Multiskan RC microplate reader (Labsystems, Thermo Scientific, Waltham, MA, USA). The optical density (OD) of each sample and standard were substracted with the OD of control wells. The VIP concentration of the samples was calculated using the standard curve.

RESULTS

1. Estrogen-dependent up-regulation of TRPA1 and TRPV1 receptor proteins in the rat endometrium

1.1. TRPA1, TRPV1 and MIF mRNA are expressed in the rat endometrium and up-regulated following estrogen treatment

Relative TRPA1 mRNA expression was significantly higher by about 8- and 2.5-fold in the endometrium of DES-treated rats compared with the untreated ones both in the sexually mature (4-month-old) and immature (4-week-old) groups. When progesterone was administered in combination with DES, this elevation was absent, and progesterone alone did not induce TRPA1 mRNA increase in either age group. Although there was an increasing tendency after ovariectomy in mature rats, TRPA1 mRNA levels did not significantly change compared with the intact controls or sham-operated animals. Similarly to TRPA1, the relative expression of TRPV1 also showed 8- and 5-fold increases in response to DES treatment in the sexually mature and immature rats respectively. After combining DES with progesterone or administering progesterone by itself, no significant change was observed in TRPV1 expression in either the young or older rats, and ovariectomy in the mature group did not significantly alter the expression levels in comparison with the intact or sham-operated group. DES induced a small, but significant 1.5- and 2.5-fold MIF up-regulation in the mature and immature rats respectively. This elevation was absent when progesterone was added to DES, and progesterone in either age group or ovariectomy in the sexually matured animals did not significantly change the expression of MIF mRNA (Statistical analysis: One-way ANOVA, Dunnett post-hoc test).

1.2. TRPA1, TRPV1 and MIF proteins are localized in rat endometrial cells and up-regulated by hormones

In the control endometrium of sexually mature rats, there is a weak to moderate TRPA1 and TRPV1 staining of the surface epithelium and glands, with scattered, weak staining of stromal cells close to the surface. Similar pattern and staining intensity was observed in the immature endometrium. MIF showed moderate to strong staining in the endometrial glands and surface epithelium, while mild positivity was detectable in the stromal cells of both age groups. In sexually mature rats, all hormone treatments and ovariectomy equally elicited significantly increased intensity of TRPA1 immunopositivity in the epithelial layer and the glands. In immature animals, DES treatment induced a considerable immunopositivity increase in the epithelium, while progesterone by itself and in combination with DES resulted in elevated

positivity in the stroma near the epithelial layer when compared with the control group. In adult rats, TRPV1 immunopositivity was observed in the stroma and the glands with weak expression in the epithelium. DES and progesterone treatment caused significantly elevated immunopositivity in all areas; the combination resulted in a similar tendency, but ovariectomy did not alter the staining intensity. In young animals, TRPV1 positivity significantly increased after DES and progesterone treatment in the stroma and the glands, while the epithelium showed weak expression (Statistical analysis: Kruskal-Wallis test, Dunn's post test).

1.3. Functional TRPA1 and TRPV1 receptors are expressed on cultured primary rat endometrial cells and their mRNA is up-regulated following DES treatment

Both the TRPA1 agonist formalin (3.3 mM) and the TRPV1 activator capsaicin (330 nM) evoked a rapid Ca^{2+} influx into the endometrial cells, providing functional evidence for the presence of functional receptor proteins. Capsaicin caused a rapid, transient Ca^{2+} influx in the cytosol of epithelial cells as detected by the magnitude of the fluorescence response. Pre-incubation of the primary rat endometrial cell cultures for 18 h with 200 pM of the non-selective ERα/ERβ agonist synthetic compound DES used in our in vivo experiments induced 10-, 17- and 5-fold up-regulation of TRPA1, TRPV1 and MIF mRNA respectively. Meanwhile, 17β-oestradiol having ERa-selective agonist potency evoked a remarkable increase (about fivefold) only in the expression of TRPV1 (Statistical analysis: One-way ANOVA, Dunnett post-hoc test).

1.4. Chronic in vivo DES pre-treatment modulates MIF-induced Trpa1 and Trpv1 expression, as well as their activation-evoked iNOS and Il1b gene expression in endometrial cells

In the primary cultures of rat endometrial cells, a 24-h incubation with 1 mg MIF/ml induced an approximately threefold up-regulation of Trpa1 mRNA, but chronic (10-day-long) in vivo DES pretreatment caused a significantly greater increase. MIF alone did not alter the expression of Trpv1 mRNA levels, but after chronic DES pretreatment, it caused a significant 2.5-fold elevation in comparison with the placebo-treated animals. TRPA1 stimulation with 3.3 mM formalin did not influence its own expression in the cells derived from the placebo-pretreated rats, but induced a significant 2.5-fold up-regulation after chronic in vivo DES pretreatment. In endometrial cells obtained from the chronically DES-pretreated rats, TRPA1 (but not TRPV1) activation induced iNOS mRNA up-regulation and both TRPV1 and TRPA1 stimulation-

resulted elevated Il1β mRNA expression (Statistical analysis: One-way ANOVA, Dunnett post-hoc test).

2. Upregulation of TRPA1 and TRPV1 ion channels in rectosigmoid DIE

2.1. TRPA1 and TRPV1 mRNA is increased in the ectopic endometrium of DIE patients

Both TRPA1 and TRPV1 were detected at the mRNA level in the normal endometrium. This clearly shows their local, not sensory neuronal expressions. qPCR measurmenets revealed differences in ectopic (rectosigmoid DIE nodule) and autologous eutopic endometrial samples (auto control endometrium) compared to normal endometrium (control). There was a remarkable 4.0–5.0 fold elevation of TRPA1 mRNA expression in the ectopic endometrium of rectosigmoid DIE lesions. We detected significantly elevated TRPV1 receptor mRNA level in both ectopic and autologous eutopic endometrium of women with endometriosis. However, the relative TRPA1 and TRPV1 expressions did not differ in the endometrium of women with sole DM or intact sigmoid bowel wall of DIE patients (Statistical analysis: Mann-Whitney U-test).

2.2. TRPA1 and TRPV1 immunoreactivity is upregulated in the ectopic endometrium of DIE patients

Scattered cytoplasmic TRPA1 and TRPV1 receptor immunostaining was detected in stromal and epithelial cells of the normal endometrium. TRPV1 labelling was sparser compared to TRPA1. Remarkable intracellular TRPA1 and TRPV1 positivity was identified in both tissue compartments of the DIE samples. Similarly to the normal endometrium, here the glandular epithelial layer was stained more vigorously. In some ectopic endometrial sections, macrophages and endothelial cells were intensely positive for both receptors, while myenteric intramural ganglia and plasmocytes of the colonic stroma showed more intensive immunoreactivity for TRPA1 than for TRPV1. Significantly increased epithelial TRPA1 protein expression was found in the DIE samples compared to the control group. Moreover, 50% increase was detected in DIE epithelium compared to DIE stroma. The TRPV1 protein expression was significantly higher both in the epithelium and stroma of the DIE patients compared to the control samples and also showed significantly increased immunopositivity (>50%) in the DIE epithelium (Statistical analysis: One-way, Bonferroni post test).

2.3. Correlation of TRPA1 and TRPV1 immunopositivity in the ectopic endometrium of DIE patients with the clinical severity

There was strong positive correlation between DM severity and stromal TRPA1 and TRPV1 immunoreactivities, the severity of dyspareunia and TRPV1 expression on ectopic epithelial cells and macrophages. Epithelial TRPA1 and stromal TRPV1 immunopositivity significantly correlated with the severity of dyschezia. We did not detect any correlation between DIE-associated painful symptoms and endothelial TRPA1 and TRPV1 immunopositivity Statistical analysis: Pearson or Spearmann correlation co-efficiencies).

3. Examination of PACAP in the sheep udder and milk, and in the lamb plasma after suckling

3.1. Concentrations of PACAP38-LI in the plasma and milk of sheep

PACAP38-LI was approximately 20 fmol/mL in the plasma of lambs before suckling, which showed an almost 2-fold increase 1 h later and returned to basal value 2 h after suckling. There was no significant difference between PACAP38 levels obtained from the jugular and mammary vein plasma samples. Plasma PACAP38-LI concentrations were similar in the three age groups. PACAP38 concentration in the milk whey of ewes was about 10 times higher as compared to the plasma samples taken from the jugular and mammary veins, but we did not detect changes in PACAP excretion in milk during the daily cycle (Statistical analysis: One-way ANOVA, Dunnett post-hoc test).

3.2. Expression of PACAP mRNA and alteration of peptide levels in the mammary gland

The relative gene expression of PACAP was similar in all the three age groups, but PACAP38-LI showed more than 2-fold increase in the 3-year-old ewes as compared to the lambs. This level remained high in the 10-year-old group, but showed greater individual differences. In comparison, the PACAP-related peptide, VIP was not expressed in the mammary gland of any age group, as shown by the lack of the presence of its mRNA measured by semi-quantitative PCR. The validity of our method was confirmed by the detection of VIP transcripts with both sets of primers in all age groups in the sheep uterus, as a positive control (Statistical analysis: Mann-Whitney U-test).

3.3. Age-related PAC1 receptor and BDNF mRNA expressions and alterations of the mammary gland

The expression of PAC1 receptor mRNA followed a gradually increasing tendency without any significant differences between the lambs, 3-year-old and 10-year-old sheep. BDNF decreased with age: the mRNA levels were strongly down-regulated by 30% and 60% in 3- and 10-year-old sheep, respectively, as compared to lambs. However, no correlation was found between BDNF and PACAP or PAC1 gene expression ratios in any age group. VPAC1/VPAC2 mRNA could not be detected either in the udder or in the uterus tissues using the primers exclusively available for sheep (Statistical analysis: Mann-Whitney U-test).

3.4. No change of VIP concentration in the plasma and milk in lactating ewes and lambs

VIP concentration in suckling lambs was approximately 20 pg/mL in lambs before suckling and in contrast to PACAP it did not change either 1 h or 2 h after suckling. In lactating ewes, we measured almost 2-fold higher peptide concentration compared to mammary and jugular vein plasma samples. Plasma VIP content was not altered during ageing, it was similar in the 3-month-old lambs, 3-year-and in 10-year-old sheep (Statistical analysis: One-way ANOVA, Dunnett post-hoc test).

CONCLUSIONS AND SUMMARY OF THE NOVEL FINDINGS

In the first part of our work we provided new data on the estrogen-dependent up-regulation and functionality of TRPA1 and TRPV1 receptors in correlation with the cytokine MIF in the rat endometrium. The endometrium undergoes continuous dynamic changes, such as proliferation, differentiation, regeneration and disintegration, in response to fluctuating estrogen and progesterone levels modulated by peptide hormones, growth factors and cytokines produced locally by various cell types [26], [27]. The well-established morphological and functional changes during the different phases of the hormonal cycle result from complex gene transcription alterations responsible for divergent molecular pathways, which are not yet fully understood.

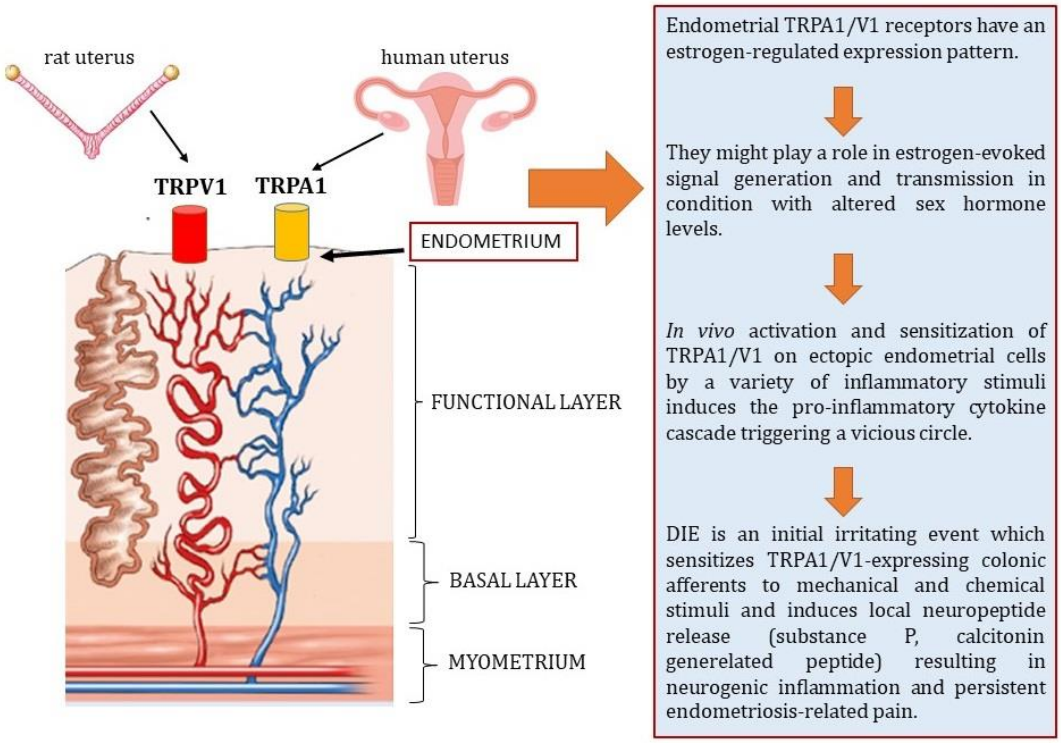
The first part of the study reports Trpa1 mRNA expression in the rat endometrium, and hormone-dependent up-regulation of TRPA1 and TRPV1 in correlation with the inflammatory cytokine MIF in both sexually immature and mature animals. The estrogen-induced TRPA1, TRPV1 and MIF increase suggests their potential roles in normal endometrial functions during the reproductive cycle, which might also be extrapolated to pathological conditions with pain and inflammation [28], [29]. We describe an estrogen-specific up-regulation of Trpa1/Trpv1 mRNA in the endometrium and also provide evidence for the functionality of these receptor proteins in cultured primary endometrial cells. Specific TRPA1/TRPV1 activation provoked an up-regulation of respective receptor mRNA in endometrial cell cultures. This can be a further evidence for receptor functionality, but a compensatory reaction to receptor desensitization cannot be excluded [30]. MIF administration also increased Trpa1/Trpv1 mRNA levels. Specific up-regulation of Trpv1 transcripts in isolated human leukocytes has been described in response to elevated serum MIF levels in a case study [31], but we demonstrate the first functional evidence of MIF–TRP signalling augmented by the chronic estrogen effects. Specific TRPA1/TRPV1 stimulation resulted in the elevated levels of iNOS and Il1b mRNA, pointing to functional relevance of endometrial TRP channels. Based on literature data obtained in keratinocytes, synoviocytes, endometriosis cells and placenta [4], [6], [32], IL1α and IL1β, NO, prostaglandin E2, substance P, IL8 and matrix metalloproteinase-1 release can be proposed from endometrial cells upon TRPA1/TRPV1 activation/sensitization by estrogen. Beside their involvement in pain/inflammation, these mediators play physiological roles in reproductive, reparative and inflammatory-like processes of the normal endometrium [33], [34].

Our results provide the first evidence that i) functional non-neuronal TRPA1 and TRPV1 receptor proteins are expressed in the rat endometrium, ii) their expressions are regulated by estrogen and positively correlate with the estrus cycle-dependent regulatory factor MIF, iii) MIF significantly increases TRPA1 and TRPV1 expression and iv) chronic estrogen action increases TRPA1/TRPV1 activation-induced iNOS and Il1b mRNA up-regulation being involved in endometrial homeostasis.

In the second part of our work we provide the first evidence on the presence of TRPA1 receptor at mRNA and protein levels in the human endometrium and its up-regulation, alongside with the TRPV1 receptor in DIE nodules of the rectum and sigmoid colon. Moreover, TRPA1 and TRPV1 expressions show correlations with the severity of many DIE-related pain symptoms, including DM, dyspareunia and dyschezia. Local inflammation and sensory neuronal sprouting play a key role in the pathogenesis of endometriosis related pain, which is mediated by a broad range of pro-inflammatory molecules. These stimulate TRPV1/TRPA1 activity both on sensory nerve terminals and non-neuronal structures, which in turn further trigger the pain. Despite ubiquitous TRPA1 and TRPV1 mRNA expressions in all the investigated tissues, significant receptor upregulation is limited to the DIE samples.

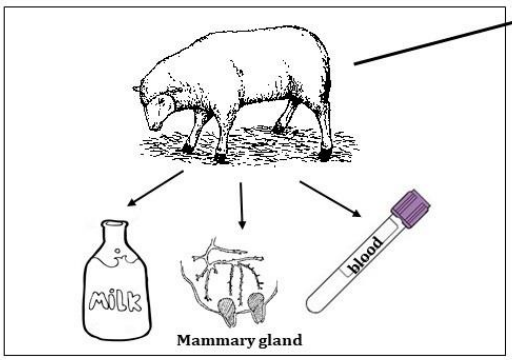
Similarly, we observed elevated TRPV1 mRNA in the eutopic endometrium of endometriosis patients as compared to the endometrium of healthy women. These observations are in agreement with recent findings showing elevated TRPV1 mRNA expression in endometriosis lesions. We believe that the increased TRPA1 and TRPV1 immunoreactivity in the stromal and most epithelial cells of the rectosigmoid DIE samples, as well as the positive correlation between their expression and the severity of painful symptoms suggests a TRPA1/TRPV1-driven sensory function for these non-neuronal cells. In the present study, the non-neuronal TRPA1 expression was more pronounced than TRPV1 in both. Immunohistochemical staining of TRPV1 receptor in healthy eutopic endometrium and in rectosigmoid DIE nodules. Despite a great deal of recent attention, there is little evidence about TRPA1 in painful gynecological conditions. Except the unaffected peritoneum adjacent to pEL lesions, TRPA1 mRNA was similar in the ectopic endometrium of pEL and the peritoneal tissue of healthy controls. Elevated TRPA1 protein expression increased in tissues with increased mechanical stress. Therefore, distortions of bowel anatomy through adhesions might contribute to the local upregulation of TRPA1 in DIE samples. Stromal TRPA1 and TRPV1 immunoreactivities strongly correlated with DM severity, as well TRPV1 expression on ectopic epithelial cells and macrophages with dyspareunia. Epithelial TRPA1 and stromal TRPV1 immunopositivity also positively correlated with dyschezia severity. Accumulating evidence supports a potential

causal relationship between endometriosis and these functional pain symptoms, viscero-visceral sensitization and neurogenic inflammation might be a plausible explanation for these frequently coexisting conditions. In summary, this is the first evidence for TRPA1 expression in the healthy human endometrium and its upregulation – alongside with that of TRPV1 – in rectosigmoid DIE nodules. Their expression increase positively correlates with the severity of different related pain symptoms suggesting their roles in the pathophysiological mechanisms.



Summary of the recent findings I: The role of non-neural TRPA1 and TRPV1 ion channels in rat and human endometrium, as well as in deep infiltrating endometriosis

The third part of our study we provide the first evidence for PACAP38 but not the related peptide VIP expression in the mammary gland of lactating ewes, its high concentration in the milk independently of the diurnal rhythm, and its rapid increase of the lamb plasma 1 h after suckling. PACAP is present in a constantly high level in the human breast milk until the 10th month after delivery and increases thereafter, between the 11th and 17th months of lactation [16], but during an actual suckling PACAP38-LI did not differ in foremilk compared to hindmilk [14]. The importance of PACAP in the cow milk is also highlighted because cow milk based formulas are used when breast milk is not available for the newborns. Our previous measurements revealed that formula contains nearly the same amount of PACAP as pasteurized cow milk and human breast milk [35]. Although the source of PACAP in the milk is not known, we showed significantly increased plasma PACAP concentration 1 h after suckling, which decreased close to the basal level 2 h after. The explanation for this finding might be a rapid absorption of PACAP38 from the milk in the proximal part of the gastrointestinal tract in lambs and/or suckling-induced PACAP release from the brain or peripheral tissues. We detected high amount of VIP in the sheep milk whey compared to the plasma, similarly to previous findings in human milk. We also showed the lack of VIP-specific mRNA in the udder in sheep, in accordance with formerly observed in rats [36]. Based on our data, we propose that the VIP content of milk derived from the maternal circulation. Furthermore, besides VIP, we did not detect any specific mRNA in these samples for VPAC 1/2 receptors either. However, this result may be due to technical issues, since there is only predicted and partial coding sequences for the sheep VPACR 1/2 available in the databases, and it is possible that this record doesn't have all the necessary start or stop sequences. We observed significant BDNF mRNA down-regulation both in 3- and 10-year-old sheep, but there were no correlations between BDNF and either PACAP or PAC1 receptor gene expressions. These data suggest that despite the brain, BDNF expression is not directly PACAP-dependent in the mammary gland during proliferation and differentiation at the developing stage. The major novelty of the present paper is that we provide the first data for a rapid increase of PACAP38 in the lamb plasma after suckling with the help of highly sensitive and specific analytical methods. The limitation of the results is that the source and the role of PACAP is not possible to experimentally access in small ruminants, therefore only hypothesis can be suggested. However, the present results clearly open new perspectives for elucidating the function of PACAP in the developing newborns, as well as a local role in the mammary gland.



PACAP

PACAP is present both at mRNA and peptide level in the mammary gland.

The high amount of this peptide accumulated in the milk might be synthesized in the mammary gland or secreted from the mother's blood.

we propose a rapid absorption of PACAP38 from the milk and/ or its release in suckling lambs

PACAP is suggested to have differentiation or proliferation promoting or immunomodulatory effects in the newborns and/or a local function on the mammary gland.

Summary of the recent findings II: Potential role of PACAP in the mammary gland of lactating ewes and lambs.

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PUBLICATIONS

1. Publications related to the thesis:

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Noémi Bohonyi*, Krisztina Pohóczky*, Bálint Szalontai, Anikó Perkecz, Krisztina Kovács, Béla Kajtár, Lajos Orbán, Tamás Varga, Sarolta Szegedi, József Bódis, Zsuzsanna Helyes, Miklós Koppán (2017) Local upregulation of Transient Receptor Potential Ankyrin 1 (TRPA1) and Vanilloid 1 (TRPV1) ion channels in rectosigmoid deep infiltrating endometriosis. MOLECULAR PAIN, (IF: 3,533; Q1; * Co-first author).

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Gabriella Gulyás, Béla Béri, András Jávor, László Márk, Éva Csősz, Krisztina Pohóczky, Beáta Soltész, Dániel Kuti, Levente Czeglédi (2012) Holtein-Fríz tehenek hosszú hasznos élettartamának vizsgálata proteomikai módszerekkel. Debreceni Egyetem Agrártudományi Közlemények, Acta Agraria Debreceniensis.

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