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

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Abstract

Transient receptor potential ankyrin 1 (TRPA1) and vanilloid 1 (TRPV1) receptors expressed predominantly in sensory nerves are activated by inflammatory stimuli and mediate inflammation and pain. Although they have been shown in the human endometrium, their regulation and function are unknown. Therefore, we investigated their estrogen- and progesterone-dependent alterations in the rat endometrium in comparison with the estrogen-regulated inflammatory cytokine macrophage migration inhibitory factor (MIF). Four-week-old (sexually immature) and four-month-old (sexually mature) female rats were treated with the non-selective estrogen receptor (ER) agonist diethylstilboestrol (DES), progesterone and their combination, or ovariectomized. RT-PCR and immunohistochemistry were performed to determine mRNA and protein expression levels respectively. Channel function was investigated with ratiometric $[Ca^{2+}]_i$ measurement in cultured primary rat endometrial cells. Both TRP receptors and MIF were detected in the endometrium at mRNA and protein levels, and their localizations were similar. Immunostaining was observed in the immature epithelium, while stromal, glandular and epithelial positivity were observed in adults. Functionally active TRP receptor proteins were shown in endometrial cells by activation-induced calcium influx. In adults, *Trpa1* and *Trpv1* mRNA levels were significantly up-regulated after DES treatment. TRPA1 increased after every treatment, but TRPV1 remained unchanged following the combined treatment and ovariectomy. In immature rats, DES treatment resulted in increased mRNA expression of both channels and elevated TRPV1 immunopositivity. MIF expression changed in parallel with TRPA1/TRPV1 in most cases. DES up-regulated *Trpa1*, *Trpv1* and *Mif* mRNA levels in endometrial cell cultures, but 17β -oestradiol having ER α -selective potency increased only the expression of *Trpv1*. We provide the first evidence for TRPA1/TRPV1 expression and their estrogen-induced up-regulation in the rat endometrium in correlation with the MIF.

Keywords

- ▶ transient receptor potential ankyrin 1 and vanilloid 1
- ▶ macrophage migration inhibitory factor
- ▶ rat endometrium
- ▶ estrogen
- ▶ progesterone
- ▶ mRNA expression
- ▶ immunohistochemistry
- ▶ fluorescent calcium imaging

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Introduction

Transient receptor potential ankyrin 1 (TRPA1) and vanilloid 1 (TRPV1) are non-selective cation channels predominantly localized in capsaicin-sensitive peptidergic sensory neurons and mediate pain and inflammation (Szallasi *et al.* 2007). TRPV1 or 'capsaicin receptor' is activated by noxious heat ($>43^{\circ}\text{C}$), protons ($\text{pH}>5.5$), bradykinin, lipoxygenase products and anandamide produced during inflammation and tissue injury. TRPV1 is also expressed in the CNS (Szolcsanyi 1988, Mezey *et al.* 2000) and in several non-neuronal cells in the skin, kidneys, lungs, testis, pancreas, spleen, cornea and uterus (Rocha *et al.* 2011, Liu *et al.* 2012, Song *et al.* 2012). Although the physiological/pathophysiological relevance of non-neuronal TRP is unknown, a cross-talk has been proposed between non-neuronal and neuronal TRP channels (Denda *et al.* 2010, Assas *et al.* 2014). Activation of non-neuronal TRPV1 in various cells results in mediator release with both physiological and pathological functions (Fernandes *et al.* 2012): activation of epidermal TRPV1 increases IL8 and prostaglandin E_2 (Southall *et al.* 2003), while TRPA1 induces IL1A and IL1B release (Nilius & Szallasi 2014).

TRPA1 is also activated by various chemical and physical stimuli, such as noxious cold ($<17^{\circ}\text{C}$), allyl-isothiocyanate and cinnamaldehyde, as well as endogenous ligands including hydrogen peroxide, formaldehyde, methylglyoxal and acrolein produced during inflammation and tissue damage (Nagata 2007). 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 in enterochromaffin cells, synovocytes, fibroblasts, melanocytes, pancreatic β -cells, epidermal keratinocytes, intestinal epithelial cells and macrophages, as well as human endometrial cells besides sensory neurones (Fernandes *et al.* 2012, Greaves *et al.* 2014).

Macrophage migration inhibitory factor (MIF) was originally identified as a T-cell-derived lymphokine (David 1966); it regulates immune responses and pain, but its specific receptor has not yet been identified. It is constitutively expressed by macrophages and T cells (Nishihira 2000), neurons (Bacher *et al.* 1998), vascular endothelial cells and smooth muscle cells (Lin *et al.* 2000). A hormonal cycle-dependent expression of MIF was described in the human endometrium, which was elevated during the late proliferative and early secretory phases predominantly driven by estrogen (Yang *et al.* 2000, Kats *et al.* 2005, Akoum *et al.* 2006). MIF is also involved in human ectopic endometrial cell proliferation (Yang *et al.*

2000), and has been proposed a biomarker of endometriosis (Akoum *et al.* 2006). The two estrogen receptor (ER) subtypes $\text{ER}\alpha$ and $\text{ER}\beta$ respond similarly to some ligands, but there are also several receptor-specific activators and responses. These receptors are located in the nucleus and regulate the transcription of specific target genes by binding to associated DNA regulatory sequences. In the uterus, $\text{ER}\alpha$ is present similarly to the mammary gland, ovary and bone. By contrast, $\text{ER}\beta$ is found mainly in the prostate, bladder, ovary, colon, adipose tissue and the immune system. Both subtypes are markedly expressed in the cardiovascular and CNSs (Paterni *et al.* 2014). The synthetic compound diethylstilboestrol (DES) is a non-selective agonist, while 17β -oestradiol has an $\text{ER}\alpha$ -selective agonist potency (Barkhem *et al.* 1998).

TRP channels have recently been described in the human endometrium, accompanied with estrogen/progesterone-induced decidualization, where the TRP canonical 1 (TRPC1) channel has been shown to be up-regulated *in vitro* (Kumar *et al.* 2014). A member of the TRPV subfamily, TRPV6, is also present in the luminal and glandular epithelia of the human, rat, mouse and pig endometrium. TRPV6 has a cycle-dependent expression pattern in the mouse and human endometrium, and the maximal amount of *Trpv6* mRNA was detected during the proliferative phase, or oestrus in the presence of high estrogen levels (Kumar *et al.* 2014).

Although TRPV1 and TRPA1 have been shown in the human eutopic and ectopic endometrium, their regulation and functional importance are not known (Wu *et al.* 2010, Liu *et al.* 2012, Song *et al.* 2012). Furthermore, there are no data regarding the link between ER 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 ER-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 the MIF.

Materials and methods

Ethics

All experimental procedures were carried out according to the 1998/XXVIII Act of the Hungarian Parliament on

Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988) and complied with the recommendations of the International Association for the Study of Pain. 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).

Animals and treatments

Four-week-old and four-month-old female Wistar rats were bred and kept in the Laboratory Animal House of the Department of Pharmacology and Pharmacotherapy, University of Pécs. They were kept with a ratio of 12 h light:12 h darkness at 24–25 °C and had access to food and water *ad libitum*. Rats of both ages were implanted with s.c. bee wax pellets containing the synthetic estrogen analogue DES (100 µg, ~2 µg daily release), or were administered daily 2 mg s.c. progesterone (Sigma–Aldrich) dissolved in sesame oil for three consecutive days ($n=5$ /group) (Garai & Clark 1992). 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; solvent-treated/sham-operated animals of the same age were used as controls.

Tissue preparation and analysis

Animals were killed under deep anaesthesia (sodium thiopental, 50 mg/kg i.p.; Sandoz, Kundl, Austria) and the uterus horns were harvested. The endometrium was scraped from the myometrium: one part was placed in RNA-Later solution (Sigma–Aldrich) and stored at –80 °C; the other one was fixed in 4% (v/v) paraformaldehyde, embedded in paraffin and sectioned for immunohistochemical analysis.

RNA extraction and quantitative real-time PCR

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). First-strand cDNA synthesis was carried out with 1 µg of total

RNA/sample using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) with oligo(dT)₁₈ primers. Relative gene expression ratios were measured with Stratagene Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA), using peptidyl-prolyl *cis-trans* isomerase A (*Ppia*) as the reference gene. Each reaction contained 2 µl of cDNA, 10 µl Luminaris Color HiGreen Low ROX qPCR Master Mix (Thermo Scientific), 0.3 µM primer and 6.8 µl water. The following primer pairs were used to amplify the target loci: *Trpa1* (sense): 5-AGTGGCAATGTGGAGCGATA-3 and (antisense): 5-TC CCGTCGATCTCAGCAATG-3; *Trpv1* (sense): 5-AATAC ACCATCGCTCTGCT-3 and (antisense): 5-CAATGTGCA GTGCTGTCTGG-3; *Mif* (sense): 5-CACCATGCCTA TGTCATCGTGAACA-3 and (antisense): 5-GCCCGGG CTCAAGCGAAGGTGGAACCGGT-3 (Sun *et al.* 2005); inducible nitric oxide synthase (*iNOS* (*Nos2*)) (sense): 5-CCAAGATGGCCTGGAGGAAC-3 and (antisense): 5-TGATGCAGTGCTACAGCTCC-3; interleukin 1 beta (*Il1b*) (sense): 5-CCTATGTCTTGCCCGTGGAG-3 and (antisense): 5-CATCCCACGAGTCACAGAGG-3; *Ppia* (sense): 5-GCAGACAAAGTTCCAAAGACAG-3 and (antisense): 5-CCATTATGGCGTGTGAAGTC-3. Amplification was carried out under the following conditions: 95 °C (10 min), followed by 40 cycles of 95 °C (15 s), 60 °C (45 s) and 72 °C (45 s). Measurements included a dissociation curve analysis to verify amplification specificity. Reactions were carried out in triplicate; relative expression ratios were calculated using MxPro QPCR Software (Agilent Technologies) with $\Delta\Delta C_t$ method, using samples of untreated animals as a calibrator. Primer efficiencies were taken into account when calculating gene expression ratios (Pfaffl 2001).

Immunohistochemistry

Paraformaldehyde-fixed, paraffin-embedded samples were deparaffinized, rehydrated and incubated in acidic citrate buffer (pH 6) in a microwave oven for antigen recovery, and then treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After washing, the sections were incubated in normal blocking solution and 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. Incubation was performed with the EnVision system anti-rabbit secondary antibody conjugated with HRP (DakoCytomation, Carpinteria, CA, USA) and the reaction was visualized using 3,3-diaminobenzidine tetrachloride containing 0.01% hydrogen peroxide

(Kun *et al.* 2012). Histological counterstaining was performed with haematoxylin. Semi-quantitative scoring of TRPA1/TRPV1 and MIF immunopositivity was performed by an expert pathologist blinded to the study. Incubating untreated rat endometrial cells with Tris-buffered saline instead of the primary antibodies served as the negative control. Sections of rat trigeminal ganglia showing a high expression of TRPA1 and TRPV1 channels were used as positive controls. Representative images of each slide were captured with a research microscope system fitted with a digital camera (Olympus BX51). TRPA1, TRPV1 and MIF immunostaining intensity was 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). The selectivity of the antibodies was proven by the lack of staining after the respective blocking peptide and literature data (Potolicchio *et al.* 2003) respectively. The endometrial thickness was measured in five different sections of each animal and then averaged in all the experimental groups.

Primary cultures of rat endometrial cells

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. Four-week-old

Wistar rats were decapitated under ether anaesthesia. Uteri were placed and cut into small strips in sterile incomplete Hank's balanced salt solution (HBSS, Ca^{2+} and Mg^{2+} free, pH 7.4) containing 1 M HEPES, 2% antibiotic-antimycotic solution and 1 M sodium hydroxide. Samples were placed in 10 ml HBSS containing 0.1% collagenase and 0.005% DNaseI and incubated at 37 ° for 30 min, and the dispersed cells were digested with 0.01% trypsin and 0.005% DNaseI at 37 ° for 5 min. Pellets were washed three times with HBSS and cultured in enriched DMEM (high glucose) containing 10% fetal bovine serum, 2% antibiotic-antimycotic solution and 0.004% kanamycin (Kornyei *et al.* 2001). All chemicals were purchased from Sigma-Aldrich. Cells were pre-incubated for 18 h with the physiological concentration of 200 pM 17β -oestradiol (Herbison 2009) having relative selectivity to ER α , and the same concentration of the non-selective ER α and ER β receptor agonist DES was used in our *in vivo* experiment (Barkhem *et al.* 1998).

After the calcium imaging measurements, cells were isolated from the plate by trypsin-EDTA diluted in incomplete HBSS, centrifuged at 700 *g* for 5 min, and then the pellet was resuspended in 600 μl RNA-Later solution (Sigma-Aldrich). Total RNA was isolated with TRI Reagent the same way as from the endometrial tissues. After DNase I digestion, the first-strand cDNA synthesis was performed from 5 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

To evaluate the effects of chronic DES pretreatment in our *in vitro* cell culture, test the functionality of the TRP

Figure 1

Relative gene expression of (A and D) *Trpa1* and (B and E) *Trpv1* receptors, as well as (C and F) *Mif* in the hormone-treated adult (A, B and C) and young (D, E and F) rat endometrium ($n=5$). Fold change represents respective mRNA levels normalized to *Ppia* as a housekeeping gene

following the $\Delta\Delta\text{CT}$ method. Each column represents the ratio of relative gene expression (mean $\pm$ s.e.m.; one-way ANOVA with Dunnett's multiple comparison test: * $P < 0.05$, *** $P < 0.0001$).

receptors, and find a direct link between the MIF cytokine and TRPA1/TRPV1, we made primary endometrial cell cultures from DES-pretreated (100 µg, w/~2 µg 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 µg MIF/µl, 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.

Ratiometric technique of $[Ca^{2+}]_i$ measurement

Sub-confluent phase of the cultured endometrial cells was determined with phase-contrast microscopy on day 7. Uterine cells were digested with trypsin and placed in lysine-covered 24-well plates before the intracellular calcium ($[Ca^{2+}]_i$) measurement. Cultures were stained with the fluorescent Ca^{2+} indicator dye fura-2-AM, as described in detail previously (Szoke *et al.* 2010). $[Ca^{2+}]_i$ 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).

Statistical analysis

Values are expressed as mean $\pm$ S.E.M. of *n*=5/group. For evaluating gene expression, analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test. For the analysis of the semi-quantitative scoring of the immunohistochemical results, the non-parametric Kruskal–Wallis test with Dunn's post-test was used. *P*≤0.05 was considered statistically significant.

Figure 2

Representative photographs of TRPA1 receptor-immunopositive areas in the rat endometrium. Immunostaining of (A) the untreated group compared with (B) DES-implanted, (C) progesterone-treated, (D) DES- and progesterone-treated and (E) ovariectomized groups, and (F) semi-quantitative scoring of TRPA1 immunopositivity in 4-month-old animals.

Box plots with the whiskers represent the medians $\pm$ 25–75 percentiles of the score values (Kruskal–Wallis with Dunn's post-test: **P*<0.05, ***P*<0.005). Magnification: 200 $\times$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0184>.

Results

Trpa1, *Trpv1* and *Mif* mRNA are expressed in the rat endometrium and up-regulated following estrogen treatment

To quantify the expression of TRPA, TRPV1 and MIF in the endometrium, we measured their relative mRNA levels by qPCR compared with the housekeeping gene *Ppia* showing the most stable expression in the uterus independently of age (Rajat *et al.* 2013; Supplementary Figure 1, see section on supplementary data given at the end of this article).

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; Fig. 1A) and immature (4-week-old; Fig. 1D) 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 (Fig. 1A and D). Although there was an increasing tendency after ovariectomy in mature rats, *Trpa1* mRNA levels did not significantly change compared with the intact controls (Fig. 1A) or sham-operated animals (fold change 1.21).

Similarly to *Trpa1*, the relative expression of *Trpv1* also showed eight- and fivefold increases in response to DES treatment in the sexually mature (Fig. 1B) and immature

(Fig. 1E) 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 (Fig. 1B and E) or sham-operated group (fold change 1.09).

DES induced a small, but significant 1.5- and 2.5-fold *Mif* up-regulation in the mature (Fig. 1C) and immature (Fig. 1F) 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 (Fig. 1C and F; sham-operated 1.19-fold).

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 staining of the surface epithelium and glands, with scattered, weak staining of stromal cells close to the surface (Fig. 2A). Similar pattern and staining intensity was observed in the immature endometrium (Fig. 3A). The mature endometrium displayed weak TRPV1 labelling restricted mostly to the epithelium (Fig. 4A). The immature endometrium showed

Figure 3

Representative photomicrographs of TRPA1-labelled (A) control, (B) DES-treated, (C) progesterone-implanted and (D) DES- and progesterone-treated groups, and (E) semi-quantitative scoring of the sections in sexually immature 4-week-old rats. Box plots with the

whiskers represent the medians $\pm$ 25–75 percentiles of the score values (Kruskal–Wallis with Dunn's post-test: $*P < 0.05$). Magnification: 200 $\times$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0184>.

Figure 4

Representative photographs of TRPV1-labelled (A) control, (B) DES-treated, (C) progesterone-implanted, (D) DES- and progesterone-treated and (E) ovariectomized groups, and (F) semi-quantitative scoring of the sections in sexually mature 4-month-old rats. Box plots with the whiskers represent

the medians $\pm$ 25–75 percentiles of the score values (Kruskal–Wallis with Dunn's post-test: $*P < 0.05$). Magnification: 200 $\times$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0184>.

no or minimal staining in the epithelial structures and no staining in the endometrial stroma (Fig. 5A). 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 (Figs 6A and 7A).

In sexually mature rats, all hormone treatments and ovariectomy equally elicited significantly increased intensity of TRPA1 immunopositivity in the epithelial layer and the glands (Fig. 2). 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. However, only the combination treatment resulted in a statistically significant immunostaining intensity increase (Fig. 3).

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 (Fig. 4). In young animals, TRPV1 positivity significantly increased after DES and progesterone treatment in the stroma and the glands, while the epithelium showed weak expression (Fig. 5).

Weak MIF expression detected in the epithelial layer and the stroma in control adult rats was more intensive after all the treatments, but only progesterone administration resulted in a significant increase focally in the epithelium (Fig. 6). In immature animals, the weak MIF immunopositivity detected in the epithelium and the surrounding stroma increased in all cases, but reached the level of statistical significance after progesterone and combination treatments (Fig. 7). The endometrial thickness was measured in all the experimental groups in five different sections of each animal and then averaged. There were no significant differences in any treatment groups, although the variations of the results were greater in sexually premature animals (Supplementary Figure 2, see section on supplementary data given at the end of this article). Negative control slides where the primary antibodies were not applied showed lack of immunostaining (Supplementary Figure 3).

Functional TRPA1 and TRPV1 receptors are expressed on cultured primary rat endometrial cells

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

Figure 5

Representative immunohistochemical images of TRPV1-labelled (A) control, (B) DES-treated, (C) progesterone-implanted and (D) DES- and progesterone-treated groups, and (E) semi-quantitative scoring of the sections in sexually immature 4-week-old rats. Box plots with the whiskers

represent the medians $\pm$ 25–75 percentiles of the score values (Kruskal–Wallis with Dunn's post-test: $*P < 0.05$). Magnification: 200 $\times$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0184>.

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. The percentage of capsaicin-responsive cells was 10.84% (4 out of 37), and then after a 2 min wash period, the responses were reproducible. Formalin administration caused Ca^{2+} influx, which developed after 30–60 s latency. The percentage of cells responding to formaldehyde was 13.5% (5 out of 37) (Fig. 8A and B). Original responses to the repetitive stimuli by capsaicin and formaldehyde are shown in Fig. 8C and D.

DES up-regulates *Trpa1*, *Trpv1* and *Mif* mRNA in the primary cultures of rat endometrial cells

Pre-incubation of the primary rat endometrial cell cultures for 18 h with 200 pM of the non-selective ER α /ER β 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, 17 β -oestradiol having ER α -selective agonist potency evoked a remarkable increase (about fivefold) only in the expression of *Trpv1* (Fig. 8E, F and G).

However, the intensity of the fluorescent calcium signals in the endometrial cells and the number of responsive cells to the TRPV1 agonist capsaicin or the TRPA1 agonist formalin was not altered after the pre-incubation with either DES or 17 β -oestradiol (data not shown).

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 μg MIF/ μl induced an approximately threefold up-regulation of *Trpa1* mRNA, but chronic (10-day-long) *in vivo* DES pretreatment caused a significantly greater increase (tenfold). 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.

Figure 6

Immunohistochemical analysis of MIF in the rat uterus of (A) control, (B) DES-treated, (C) progesterone-implanted, (D) DES- and progesterone-treated, (E) ovariectomized groups, and (F) semi-quantitative scoring of the sections in sexually immature 4-month-old rats. Box plots with the whiskers

represent the medians $\pm$ 25–75 percentiles of the score values (Kruskal–Wallis with Dunn's post-test: $**P < 0.005$). Magnification: $200\times$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0184>.

TRPV1 activation with 330 nM capsaicin resulted in a 30-fold increase of *Trpv1* mRNA in the cells obtained from the placebo-pretreated animals, but a significantly greater increase (40-fold) in the endometrial cell cultures of the DES pretreated ones (Fig. 9A).

In endometrial cells obtained from the chronically DES-pretreated rats, TRPA1 (but not TRPV1) activation-induced *iNOS* mRNA up-regulation was significantly decreased, but both TRPV1 and TRPA1 stimulation-evoked *Il1b* mRNA increases were significantly reduced when compared with placebo pretreatment (Fig. 9B).

Discussion

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 (von Wolff *et al.* 2000, Srisuparp *et al.* 2001). 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 present 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 (Suzuki *et al.* 1996, Akoum *et al.* 2006, Liu *et al.* 2012).

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 (Biro *et al.* 1998, Czikora *et al.* 2013). 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 (Bachiocco *et al.* 2011), but we demonstrate the first functional evidence of MIF–TRP signalling augmented by the chronic estrogen

Figure 7

Immunohistochemical analysis of MIF in the rat uterus of (A) control, (B) DES-treated, (C) progesterone-implanted and (D) DES- and progesterone-treated groups, and (E) semi-quantitative scoring of the sections in sexually immature 4-week-old rats. Box plots with the whiskers

represent the medians $\pm$ 25–75 percentiles of the score values (Kruskal–Wallis with Dunn's post-test: $*P < 0.05$). Magnification: $200\times$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0184>.

effect. Specific TRPA1/TRPV1 stimulation resulted in the elevated levels of *iNOS* and *Il1b* mRNA, pointing to functional relevance of endometrial TRP channels. These mediators play roles in the local control of the endometrium during the normal hormonal cycle, e.g. vasodilation (NO) and pro-inflammatory responses during ovulation, pregnancy or inhibition of decidualization (IL1B) (Telfer *et al.* 1997, Mizuno *et al.* 1999, Wira *et al.* 2010). Moreover, both are released from ectopic endometrial cells upon TRPA1/TRPV1 activation (Greaves *et al.* 2014). *Il1b* and *iNOS* transcripts were down-regulated after TRPA1/TRPV1 activation in the cells obtained from the DES-pretreated animals. Transcriptional responses after prolonged effects of DES can reflect an estrogen-driven signalling and underlying gene expression patterns: MIF levels are cycle-dependent in the human endometrium (Kats *et al.* 2005), *Il1b* is down-regulated (Wira *et al.* 2010), while *iNOS* is regulated by estrogen action in a complex manner (Han *et al.* 2005).

TRPV1 mediates NO and IL1B release in human endometriosis (Hucho & Levine 2007, Liu *et al.* 2012). Stimulation of TRPV1 in rat C fibres is dependent on the oestrous cycle favouring oestradiol (Peng *et al.* 2008), and oestradiol may contribute to pain through TRPV1

regulation in the hippocampus (Wu *et al.* 2010). It exerts classical (slow, genomic) effects on *Trpv* transcription (Hoenderop *et al.* 2005) and can also directly or indirectly stimulate the TRPV1 receptor (Kumar *et al.* 2014). In contrast to mRNA, progesterone also increased TRPA1 and TRPV1 protein expression levels in the endometrium, possibly due to sensory neural expression, local translational/protein degradation differences or mRNA contribution of different cell types. Progesterone is also well known to mediate β_2 -adrenergic receptor transcription (Vivat *et al.* 1992) and eventually might have an indirect effect on TRPV1 signalling, but no direct modulation has been described so far (Kumar *et al.* 2014).

Ovariectomy resulted in increasing *Trpa1/Trpv1* and *Mif* mRNA expression tendencies, and significant TRPA1 protein up-regulation in the endometrium. Reduced estrogen effects (e.g. after ovariectomy) up-regulate or stimulate TRPV1 and increase pain/hyperalgesia (Idris *et al.* 2010, Wu *et al.* 2010, Rossi *et al.* 2014). According to a well-proven explanation, high estrogen levels determine a female phenotype of signalling pathways downstream of the β_2 adrenergic receptor, such as attenuated protein kinase A and $C\epsilon$ activities (Hucho & Levine 2007). Ovariectomy alleviates suppression of these

Figure 8

(A and B) Representative transmitted light pseudocolour images of a rat endometrial cell before and after the application of the TRPV1 agonist capsaicin (330 nM). Original recordings showing the responses induced by (C) repetitive capsaicin and (D) the TRPA1 agonist formalin (3.3 mM) stimuli. Relative gene expression of (E) *Trpa1*, (F) *Trpv1* and (G) *Mif* mRNA after an 18-h administration of DES and 17β-oestradiol ($n=4$ /group).

Fold changes represent respective mRNA levels normalized to the *Ppia* as a housekeeping gene following the $\Delta\Delta CT$ method. Each column shows the ratio of relative gene expression (mean $\pm$ s.e.m.; one-way ANOVA with Dunnett's multiple comparison test: ** $P<0.005$, *** $P<0.0001$). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0184>.

protein kinases that are known to activate or sensitize TRPV1 (Goswami *et al.* 2011). This was described in neurons where reduced estrogen levels provoke pain/hyperalgesia (Idris *et al.* 2010, Wu *et al.* 2010, Rossi *et al.* 2014). Ovariectomy also increases non-neuronal TRPV1 and MIF expression (Kastin *et al.* 1992, Oshima *et al.* 2006, Emmerson *et al.* 2009, Idris *et al.* 2010, Wu *et al.* 2010, Kahnmoie 2012, Rossi *et al.* 2014). Endometrial cells express the β_2 adrenergic receptor (Ontsouka *et al.* 2004, Bruzzone *et al.* 2005); therefore, it is reasonable to explain our ovariectomy-evoked results by this nor-adrenaline-dependent system.

We demonstrated that *Mif* transcription is regulated by DES in the rat endometrium in parallel with *Trpa1/Trpv1* mRNA levels. Its expression is mediated by estrogen levels, while ovariectomy also increases MIF expression. Beside its functions in innate immunity, MIF is a potent angiogenic, anti-apoptotic, proliferative and tissue remodelling factor (Calandra & Roger 2003) partly by acting on the CD74 receptor (Leng *et al.* 2003). It is expressed cycle-dependently in human endometrial epithelial and stromal compartments (Kats *et al.* 2005).

In order to assess the involvement of ER α and ER β in the estrogen-driven up-regulation of these molecules, the

Figure 9

Relative mRNA expression of (A) *Trpa1* and (B) *Trpv1* receptors, as well as (C) *iNOS* and (D) *Il1b* in the primary endometrial cell cultures derived from chronically DES-pretreated or placebo-pretreated rats in response to (A and B) 1 μ g MIF/ μ l, (A, C and D) TRPA1 activation by 3.3 mM formalin or (B, C and D) TRPV1 stimulation by 330 nM capsaicin ($n=4$ /group).

Fold change represents respective mRNA levels normalized to *Ppia* as a housekeeping gene following the $\Delta\Delta CT$ method. Each column shows the ratio of relative gene expression (mean $\pm$ s.e.m.; one-way ANOVA with Dunnett's multiple comparison test: ## $P<0.005$, ### $P<0.0001$ vs solvent-treated cells and ** $P<0.005$, *** $P<0.0001$ vs placebo-treated group).

primary rat endometrial cell cultures were pre-incubated with the non-selective ER α /ER β agonist synthetic compound DES in comparison with 17 β -oestradiol having ER α -selective agonist potency. DES up-regulated *Trpa1*, *Trpv1* and *Mif* mRNA in this *in vitro* system, similarly to our whole tissue results following the *in vivo* treatment. Meanwhile, only *Trpv1* transcription increased in response to 17 β -oestradiol, suggesting that ER β might also play a role in the regulation of TRPA1, TRPV1 and MIF. Despite the marked up-regulation of these molecules, fluorescent calcium signal intensity and number of endometrial cells responsive to TRPV1/TRPA1 activation were not altered after pre-incubation with either DES or 17 β -oestradiol. This might be due to intracellular calcium signals being regulated by several different mechanisms including the contribution of intracellular stores; therefore, this technique is less suited for quantitative analysis. Moreover, another possible explanation is that TRP receptors in the endometrium may play a role in signal transduction pathways similarly to Na⁺/K⁺-ATPase that is involved in the assembly of multiple protein complexes transmitting signals to different intracellular compartments (Xie & Cai 2003). Further studies are needed to determine this issue and draw a conclusion on the mechanism.

Based on literature data obtained in keratinocytes, synoviocytes, endometriosis cells and placenta (Cella *et al.* 2008, Fernandes *et al.* 2012, Liu *et al.* 2012, Nilius & Szallasi 2014, Wu *et al.* 2015), IL1A and IL1B, 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 (Curry & Osteen 2003, Goff 2004, Thippeswamy 2006, Wira *et al.* 2010).

Endometrial TRPA1 and TRPV1 receptors have an estrogen-regulated expression pattern similar to dorsal root ganglion primary sensory neurons innervating the area and also other cell types (Hoenderop *et al.* 2005, Greaves *et al.* 2014). This indicates that they might play a role in estrogen-evoked signal generation and transmission in conditions with altered sex hormone levels. Similarly to other TRP or cation channels of the endometrium (Ruan *et al.* 2014, Singh *et al.* 2015), TRPA1/TRPV1 may mediate homeostatic and barrier functions, Ca²⁺ signalling, cell proliferation/differentiation, endometrial receptivity, embryo implantation and sensory-immune interactions (Fernandes *et al.* 2012). Non-neuronal TRPV1 can also be activated by the

endocannabinoid anandamide in the rat reproductive system (Cella *et al.* 2008, Scotchie *et al.* 2014).

These results are consistent with increased TRPA1 and TRPV1 expression in rat and human endometriosis demonstrated by our laboratory (unpublished results) and literature data (Hucho & Levine 2007, Ilie & Ilie 2013, Graham *et al.* 2015). Increased estrogen synthesis in endometriosis lesions makes those results comparable to these data. Furthermore, the TRP agonist endocannabinoids regulate the migration of endometrial stromal cells (Gentilini *et al.* 2010). TRPA1/TRPV1 in non-neural cells (stromal, glandular and epithelial cells) might have estrogen-mediated normal sensory, secretory and consequent inflammation-generating and pain-transmitting roles under pathological conditions. Although not investigated by the present study, non-neural TRP channels may amplify pain/inflammatory signals under pathological conditions as described in keratinocytes (Pang *et al.* 2015) and synoviocytes (Wu *et al.* 2015).

The present 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 oestrus 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.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-15-0184>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

K P, J K, B S, J G, L C and Zs H conceived and designed the experiments. K P, J K, B S, E S Z, E S, M P and A P performed the experiments. K P, A G, B K and K K analysed the data. J L K contributed reagents, materials and analysis tools. K P, J K, B S, J L K, J G and Zs H wrote the paper.

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