

Effects of selective estrogen receptor agonists on estrogen receptor expression in the uterus of ovariectomized rats

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ABSTRACT

The aim of the present study was to investigate effects of short time treatment with estrogen receptor (ER) selective agonists on ER α , ER β and G-protein coupled estrogen receptor-1 (GPER) expression in the uterus of ovariectomized rats. The rats were treated with either estradiol (E2), the specific ER α agonist PPT or the specific ER β agonist DPN for 18 hrs. Uterine weights were higher after E2 or PPT treatment than after DPN or no treatment. ER α mRNA levels decreased significantly in PPT and DPN treated animals as compared to controls. Stromal ER α immunostaining was higher after E2 treatment than in controls. The ER β mRNA level was lower in the E2 and PPT groups compared with controls. ER β immunoreactivity was higher in the myometrium after DPN treatment than in controls. The GPER mRNA level was lower in the E2 and DPN groups as compared to the controls, whereas total protein levels did not display any change. The proliferation marker Ki-67 increased after PPT treatment in stroma and myometrium, as compared to controls. Thus, uterine growth and proliferation are predominately regulated via ER α . Also ER β expression showed regulation via ER α , while GPER expression indicated control via ER β . This short term treatment did not result in any regulation of the total protein level as determined by Western blot. However, treatment by E2 increased ER α immunostaining in the stroma and DPN augmented ER β immunostaining in the myometrium. Thus, the estrogen receptors in the rat uterus are differently regulated depending on the ligand and tissue type.

Keywords: PPT; DPN; ER α , ER β , GPER

1. INTRODUCTION

Estrogen and progesterone provide the basis for the cy-

elic changes in the uterine tissues during the estrous cycle. The action of estrogen is primarily mediated via binding to specific intracellular receptors in the target cells. The estrogen receptors (ER) are members of a superfamily of nuclear transcription factors with highly homologous DNA binding and ligand binding domains [1-5]. After the discovery of the β subtype [1], the hormonal signals are now assumed to be transduced by both ER α and ER β [1,3,4]. Both ERs bind estradiol (E2) with high affinity and specificity [6]. Although ER β shares many functional characteristics with ER α , the molecular mechanisms regulating the transcriptional activity and the tissue location of ER β are distinct from those of ER α [1,6]. In ovariectomized rats, E2 induces DNA synthesis and mitosis in the uterus, whereas progesterone inhibits DNA synthesis in the epithelium, but stimulates mitosis in stromal cells [7,8]. ER α turns on target gene expression and functions as a regulator of ligand-activated transcription in estrogen responsive tissues [9].

The membrane bound estrogen receptor G-protein coupled receptor-30, now denoted G-protein coupled estrogen receptor-1 (GPER), has been described and studied intensely during the last decade [10-15]. GPER seems to be of great importance for several metabolic functions in the female mice as found by studies in GPER knock-out mice, while no effect on the reproductive tract, e.g. uterine weight, was found [13]. The uterine weight increased in GPER $-/-$ mice after treatment with E2 [16]. There has been studies claiming that GPER has no effect in the female reproductive tract [17], but also that GPER is a factor of poor prognosis of endometrial cancer [18]. Recently an article describes that GPER activation opposes estrogen dependent uterine growth via inhibition of phosphorylation signals [19], indicating why GPER still could have importance in the female reproductive tract.

The non-steroidal compounds PPT and DPN (4,4',4''-(4-Propyl-pyrazole-1,3,5-triyl) trisphenol and 2,3-bis(4-hydroxyphenyl)-propionitrile, respectively) are characterized as selective agonists for ER α and ER β , respectively. PPT is approximately 1000-fold more potent as an

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agonist on ER α than on ER β and has a 400-fold preference towards ER α in its binding affinity [20,21]. DPN has a 70-fold higher relative binding affinity and 170-fold higher relative potency in transcription assays with ER β than with ER α [22]. None of the compounds are known to bind GPER. 17 β -estradiol (E2) has equal affinity (K_d approx. 0.1 - 1 nM) for ER α and ER β [6], and a little less (K_d approx. 6 nM) to GPER [11].

The aim of the present study was to determine the distribution of ERs, also regarding subtype, in the rat uterus after short term treatment with selective estrogen receptor agonists. We have studied ER α , ER β and GPER expression and immunostaining in the uteri of ovariectomized rats treated with an ER α agonist (PPT), an ER β agonist (DPN) or E2. In addition, uterine growth and the proliferation marker Ki-67 were monitored.

2. MATERIAL & METHODS

2.1. Animals

Thirty female adult Sprague-Dawley rats, 55 - 60 days old and weighing approximately 250 g were used. The animals were housed in a controlled environment at 21°C - 23°C on an illumination schedule of 12 h of light and 12 h of darkness. Standard pellet food and water were provided ad libitum. The rats were ovariectomized (ovx) during anesthesia with Xylazin (Bayer AG, Leverkusen, Germany; 0.75 mg/100g rat) and Ketaminol (Intervet AB, Boxmeer, Netherlands; 7 mg/100g rat). They were housed for 14 days before initiation of hormone treatment. The ovx animals were treated with one single s.c. injection with 5 μ g E2/rat (n = 8), 1.25 mg PPT (4,4',4''-(4-Propyl-pyrazole-1,3,5-triyl) trisphenol)/rat (n = 8) or 3.125 mg DPN (2,3-bis(4-hydroxyphenyl)-propionitrile)/ rat (n = 6), 18hrs before sacrifice. OvX animals treated with vehicle (OvxC) served as controls (n = 8). The animal studies were approved by the Committee on Animal Care in Sweden.

2.2. Hormones

Estradiol-17 β was purchased from Sigma Co. (St. Louis, Missouri), and dissolved in 99.5% ethanol at a high concentration and then diluted with 50:50 DMSO:PBS to the proper concentration. The final concentration of ethanol was less than 2% in the injections. PPT and DPN were bought from Tocris Cookson, via Bio Nuclear, Bromma, Sweden.

2.3. Tissue Collection

At sacrifice the uterus was removed, stripped of fat and connective tissue, weighed and cut in one half and two quarter pieces. One quarter was immersion-fixed in 4% formaldehyde at 4°C for 8 hours and stored at 4°C in

70% ethanol and thereafter embedded in paraffin. One quarter of the uterine tissue was stored in -70°C for protein extraction. The rest of the tissue was placed in RNA stabilization solution (RNAlater, Ambion, Austin, TX, USA) immediately after collection and stored at -20°C until analyzed.

2.4. RNA Preparation and Reverse Transcription

Total RNA from the uterine tissue of each animal was purified with the RNeasy kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Two μ g of total RNA from each sample was reverse transcribed at 37°C for 60 min in a final volume of 20 μ l with a reaction mixture (Qiagen GmbH, Hilden, Germany) containing 1 \times RT buffer, dNTP mix (0.5 mM each dNTP), 300 ng random primers (Invitrogen, Paisley, UK), 10 units RNase inhibitor (Supersase-In, Ambion, Austin, TX), and 4U of OmniscriptTM reverse transcriptase (Qiagen).

2.5. Real Time PCR for ER α , ER β , GPER and RPLP0

Real time PCR was performed using cDNAs corresponding to 100 ng RNA either using 10 μ l of QuantitectTM SYBR[®] Green PCR mix (Qiagen) containing HotStarTaq DNA polymerase, PCR buffer, dNTP mixture and 0.3 μ M of each oligonucleotide primer in a final volume of 20 μ l or 12.5 μ l of iQTM SYBR[®] Green Supermix (Bio-Rad) and 0.3 μ M of each oligonucleotide primer in a final volume of 25 μ l. The samples were subjected to an amplification of 40 cycles. The annealing temperatures were 57°C for ER α , ER β and RPLP0, and 60°C for GPER. The purity of PCR products was confirmed by a melting curve analysis in all experiments. The oligonucleotide primers for ER α , ER β , GPER and RPLP0 are as follows: ER α (Accession number NM_01-2689.1) 5'-TACGAAGTGGGCATGATGAA-3' (forward) and 5'-AAGGTTGGCAGCTCTCATGT-3' (reverse); ER β (Accession number NM_012754.1) 5'-TATCTCTCC-CAGCAGCAGT-3' (forward) and 5'-CTCCAGCAGC-AGGTCATACA-3' (reverse); GPER (Accession number NM_133573.1) 5'-TTCATCAACCTGGCAGCGGCTG-3' (forward) and 5'-TGCAGAGCACGGCGATATCGT-3' (reverse); RPLP0 (Accession number NM_001002.3) 5'-GGCGACCTGGAAGTCCAAC-3' (forward) and 5'-CCATCAGCACCACAGCCTTC-3' (reverse) to yield products of 138, 145, 100 and 149 bp respectively. Each PCR assay included a negative control containing a RNA sample without reverse transcription. All PCR reactions were performed twice for all rats. The values of relative expression of genes of interest were normalized against the housekeeping gene RPLP0, which was selected out

of several tested housekeeping genes as an invariable internal control.

2.6. Immunohistochemistry

Immunohistochemical technique (avidin-biotin-peroxidase) was used to visualize ER α , ER β GPER and Ki-67 immunostaining intensity and distribution. A monoclonal mouse anti-human antibody was used for detection of ER α (08-1149, Zymed Laboratories, Inc., San Francisco, CA). It recognizes the N-terminal domain (A/B region) of ER α . A polyclonal rabbit anti-rat ER β antibody (PA1-310B, Affinity Bioreagents, Inc., UK), which corresponds to the C-terminal amino acid residues 467 - 485, was used for detection of ER β . An anti-human GPER polyclonal antibody (HPA027052) raised in rabbit was purchased from Atlas antibodies, Sweden. A monoclonal mouse anti-human antibody was used for detection of Ki-67 (NCL-Ki67-MM1, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK). After the tissue sections were dewaxed and rehydrated, an antigen retrieval procedure was performed. Sections were pretreated in a microwave oven at high power, in 0.01 M sodium citrate buffer (pH 6.0) for 10 min, and then allowed to cool for a further 20 min. Following washing in buffer (0.1 M Phosphate buffered saline (PBS), pH 7.6, for ER α , GPER and Ki-67, and 0.1M Tris-buffered saline (TBS), pH 7.4, for ER β), non-specific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for 10 min at room temperature. The sections were then washed for 10 min in buffer, and were blocked for 30 min in non-immune horse serum diluted in PBS for ER α and Ki-67, normal goat serum in TBS containing 5% (w/v) BSA for ER β and donkey serum diluted in PBS for GPER in a humidified chamber at room temperature. The tissue sections were thereafter incubated with the respective primary antibodies (ER α 1:5, ER β 1:1000, GPER 1:150 and Ki-67 1:200) at 4°C overnight.

Negative controls were obtained by replacing the primary antibody with non-immune IgG of the equivalent concentration. Following primary antibody binding, the sections were incubated with the appropriate second antibody, for ER α and Ki-67: a biotinylated horse anti-mouse IgG (Vectastain, Vector), diluted in normal horse serum, was used for 60 or 30 min respectively at room temperature; for ER β : a biotinylated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology, CA), diluted in normal goat serum, was incubated for 30 min at room temperature and for GPER: a biotinylated donkey anti-rabbit IgG antibody (Santa Cruz Biotechnology, CA). Thereafter the tissue sections were incubated for 30 min at room temperature with a horseradish peroxidase-avidin biotin complex (Vectastain Elite, Vector, CA). The site

of the bound enzyme was visualized by the application of 3,3'-diaminobenzidine in H₂O₂ (DAB kit, Vector, CA), a chromogen which produces a brown, insoluble precipitate when incubated with enzyme. The sections were counterstained with hematoxylin and dehydrated before mounted with Pertex®.

2.7. Image Analysis

A Leica microscope connected to a computer using Colovision software (Leica Imaging System Ltd. Cambridge, England) was used to assess ER α and Ki-67 immunostaining quantitatively by a computer image analysis system. Quantification of immunostaining was performed on the digitized images of systematic randomly selected fields of endometrial stroma, from which non-stromal elements (e.g. luminal and glandular epithelium) were interactively removed and analyzed separately. All luminal and glandular epithelia as well as 12 fields of stromal cells were measured separately in each tissue section. By using color discrimination software, the total area of positively stained cells (brown reaction product) was measured, and expressed as a ratio of the total area of cell nuclei (brown reaction product + blue haematoxylin).

2.8. Manual Scoring

The intensity and tissue distribution of ER β and GPER immunostained cells were manually evaluated independently by two observers blinded to treatment, using a semi-quantitative manual scoring on a four point scale: (-) = negative, (+) = faint, (++) = moderate and (+++) = strong immunostaining.

2.9. Protein Extraction

Uterine tissues were disrupted in ice-cold cell lysis buffer (1:10 tissue:buffer) containing 50 mM Tris-HCl, 150 mM NaCl, 1% Triton-X and 5 mM EDTA. Before use 1 tablet protease inhibitor (Roche Complete Mini protease inhibitor, Roche) was added/10 ml buffer. Protein concentrations were measured by Lowry protein assay with BSA as a standard [23].

2.10. Western Blot

Uterine samples from all rats were available for western blot. Ten μ g of protein extract from each sample were resolved on 10% polyacrylamide gels (Bio-Rad) in Mini-Protean II cell (Bio-Rad) using Tris-Glycine buffers. The proteins from the gel were transferred to a PVDF membrane (Amersham) by electroblotting. After blocking the membranes in 5% non-fat milk (NFM) in 0.1% TBS-Tween (overnight at 4°C for ER α , 60 min at RT for ER β and GPER), they were incubated (3 hours at RT for ER α

and overnight at 4°C for ER β and GPER) with primary antibodies (ER α F-10, Santa Cruz, 1:500; ER β E1276, Sigma, 1:1000; GPER HPA027052, Atlas, 1:500). Membranes were washed and incubated for 60 min at RT with HRP-conjugated secondary antibodies (1:2000, anti-rabbit for ER α and GPER or anti-mouse for ER β , Santa Cruz). Membrane was washed and incubated in Amersham ECL Plus™ Western Blotting detection reagents (GE Healthcare, UK) for 5 min., and exposed to ECL Hyperfilm (GE Healthcare, UK) and developed. Densitometric analyses of the films were performed using AlphaView software from Alpha Innotech Corporation.

2.11. Statistics

The results are presented as median and range, if not otherwise stated. Statistical analysis were performed by ANOVA on ranks (Kruskal-Wallis test) followed by Dunn's test for evaluation of significance. The results are presented as box and whisker plots, with 50% of the samples within the box. Values with an asterisk are significantly different ($p < 0.05$) to OvxC.

3. RESULTS

3.1. Uterine Weights

As an indication of the estrogenic effects of the treatments, the uterine weights were measured. The weights in grams (mean \pm SEM) increased as could be expected from the respective treatment. PPT (0.205 g \pm 0.009 g) and E2 (0.169 g \pm 0.009 g) treatments increased uterine weight significantly as compared to the OvxC (0.120 g \pm 0.007 g) group. Thus, DPN (0.120 g \pm 0.005 g) treatment had no effect on uterine weight.

3.2. ER α , ER β and GPER mRNA Levels

The uterine ER α mRNA levels, standardized to RPLP0, were decreased after all treatments as compared to OvxC, but reached significance only for PPT and DPN (**Figure 1**; **Table 1**). The ER β mRNA levels were decreased after

E2 and PPT treatment as compared to the OvxC group, whereas DPN had no effect (**Figure 1**; **Table 1**). The GPER mRNA levels decreased after all treatments but reached significance for only E2 and DPN treatments, as compared to the OvxC group (**Figure 1**; **Table 1**).

3.3. ER α , ER β and GPER Protein Levels

A representative western blot of ER α , ER β , GPER and β -actin is shown in **Figure 2**. There were no statistically significant differences found between the treatment groups (data not shown), since there was quite a variation between the individual rats. Some of the PPT treated ani-

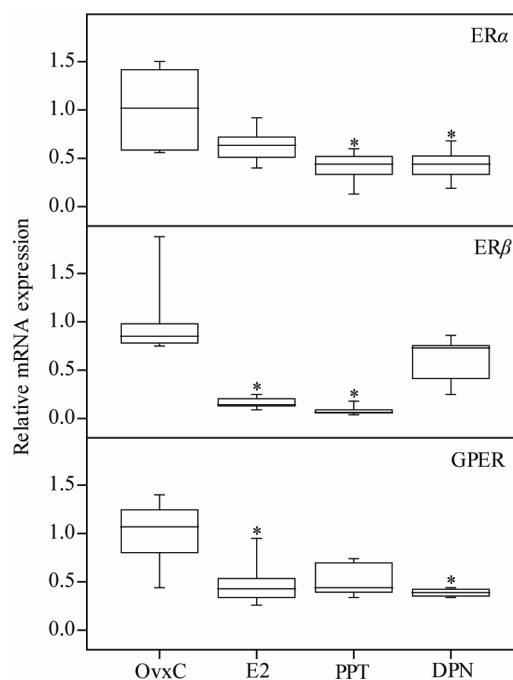


Figure 1. The mRNA levels of ER α , ER β and GPER in the rat uterus. OvxC = ovariectomized controls; E2, PPT and DPN treated rats; n = 8 in all groups but DPN where it is 6. Bars with an asterisk show significant difference to the control group (OvxC) ($p < 0.05$).

Table 1. Summary of the treatment effects on the mRNA levels and immunohistochemical staining of ER α , ER β , GPER and Ki-67 proteins. Arrows indicate the significant changes found.

	E2	PPT	DPN
IHC: ER α	↑ stroma	—	—
ER α mRNA	—	↓	↓
IHC: ER β	—	—	↑ myometrium
ER β mRNA	↓	↓	—
IHC: GPER	—	—	—
GPER mRNA	↓	—	↓
IHC: Ki-67	—	↑ stroma + myometrium	—

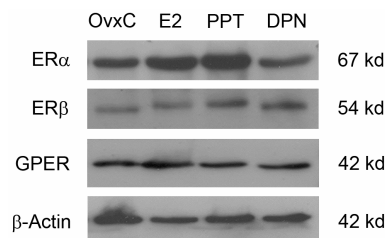


Figure 2. A representative western blot of ER α , ER β and GPER in uterus from E2, PPT and DPN treated rats and the OvxC control group. β -Actin was used as loading control.

mals exposed a stronger ER α band than the OvxC group, but others did not exhibit a difference, why there are no differences when looking at the groups.

3.4. Immunohistochemical Localization of ER α , ER β and GPER

Results from ER α immunostaining in the uterus showed that E2 treatment increased positive immunostaining in the uterine stroma as compared to the OvxC group (**Figures 3(A)-(D)**; **Figure 4**, top panel; **Table 1**). No differences were found in the myometrium, luminal and glandular epithelium (**Figure 4**). ER β immunostaining in the myometrium was increased after DPN treatment, as compared to the OvxC group (**Figures 3(E)-(H)**; **Figure 5**, bottom panel; **Table 1**). GPER immunostaining was present in membranes and cytoplasm of all the examined cell types (**Figures 3(I)-(L)**). GPER was strongly stained in myometrium and blood vessels. However, there were no significant differences in GPER immunoreactivity between the different treatment groups in any of the cell types examined (**Figures 3(I)-(L)**; scoring results not shown).

3.5. Proliferation

As assessed by Ki-67 immunostaining and image analysis in the uterus, proliferation was increased in stroma and myometrium after PPT treatment as compared to the OvxC group (**Figures 3(M)-(P)**; **Figure 6**, top and bottom panels; **Table 1**). There were no differences in proliferation between the treatment groups in luminal and glandular epithelium (**Figure 6**).

4. DISCUSSION

Estrogens exert biological effects in many tissues. The multitude of estrogen responses matches the widespread distribution of ERs throughout the body, with ER α expression being the most common isotype in e.g. uterus, pituitary, liver and kidney, while ER β expression is dominating in the ovary and prostate [6]. GPER has been found in a wide variety of tissues, e.g. in reproductive tissues such as uterus and ovary [12]. In accordance with a study on mice [24], PPT and E2 induced uterine weight similarly. Our rats were treated with E2 at a dose of 5 μ g/250 g animal and day, while the mice got 0.5 μ g/animal and day. Since the mice were 21 days and then weighing approximately 25 g, the dose/100 g animal should equal ours at 2 μ g/100 g animal. The PPT dose for the mice was 500 μ g/animal and day (2 mg/100 g mouse), while we gave 1.25 mg/250 g rat and day (0.5 mg/100 g rat). Thus, their PPT dose was higher than ours, but the results were similar. In another study on ovx rats, 75 μ g PPT (30 μ g/100 g rat) increased the uterine weight

after 24 hours [25]. Thus, the effect on uterine weight from PPT treatment is achieved with an even lower dose of PPT as well. Furthermore, proliferation was significantly induced after PPT treatment in stroma and myometrium, as compared to controls. In addition, DPN treatment neither increased uterine weight nor proliferation. Consequently, growth and proliferation are mediated primarily by ER α in the uterus, which confirms previous results [26].

E2 is known to regulate the expression of ER α at both mRNA and protein levels [27]. An earlier report on ovx rats show that ER α mRNA levels were up regulated with no change in protein levels 24 h after E2 administration [28]. Our data shows that E2 treatment causes no change in the total mRNA and protein levels but up regulates ER α protein in stroma after 18 h, indicating a time and cell type dependent regulation of the receptor. However, PPT and DPN down regulated ER α expression suggesting both ER α and ER β to be involved in regulation of ER α mRNA.

In the E2 and PPT treated groups the ER β mRNA level was down regulated, indicating that ER β expression is regulated via ER α . A previous study showed that total ER β mRNA expression was up regulated after 24 h of E2 administration [28]. Taken together, it appears that there is an initial down regulation of ER β followed by an increase in the total mRNA expression. Such initial down regulation of the ER mRNA level by E2 injections has been described before in the uteri of ovx rat [29]. The ER mRNA level was thereafter transiently up regulated but decreased again after 72 hrs [29].

Both ER α and ER β immunostaining is predominantly found in the nuclei, some staining can though be found in the cytoplasm, probably due to the fact that the receptors without ligand are also present outside the nucleus [30, 31]. Since many effects from estrogens are too rapid to be mediated via the nuclear receptors, more and more evidence have suggested the presence of a cytosolic estrogen receptor [31]. GPER is one receptor, which has been described as an estrogen receptor present in membranes and cytoplasm [10].

Interestingly, we observed that both E2 and DPN down regulated GPER mRNA. A recent report shows that E2 down regulates GPER mRNA in MCF-7 cells [32]. Our data suggests that the regulation of GPER mRNA levels is mediated predominantly via ER β in the rat uterus. The discrepancy between down regulated mRNA levels from PCR assays and unaffected protein levels in Western blots could be due to e.g. microRNAs, which are known to regulate processes at the transcriptional and translational level [33]. The mRNA determinations and Western blots are performed in a tissue homogenate, which is different to the immunostaining results obtained by scoring the different tissue parts

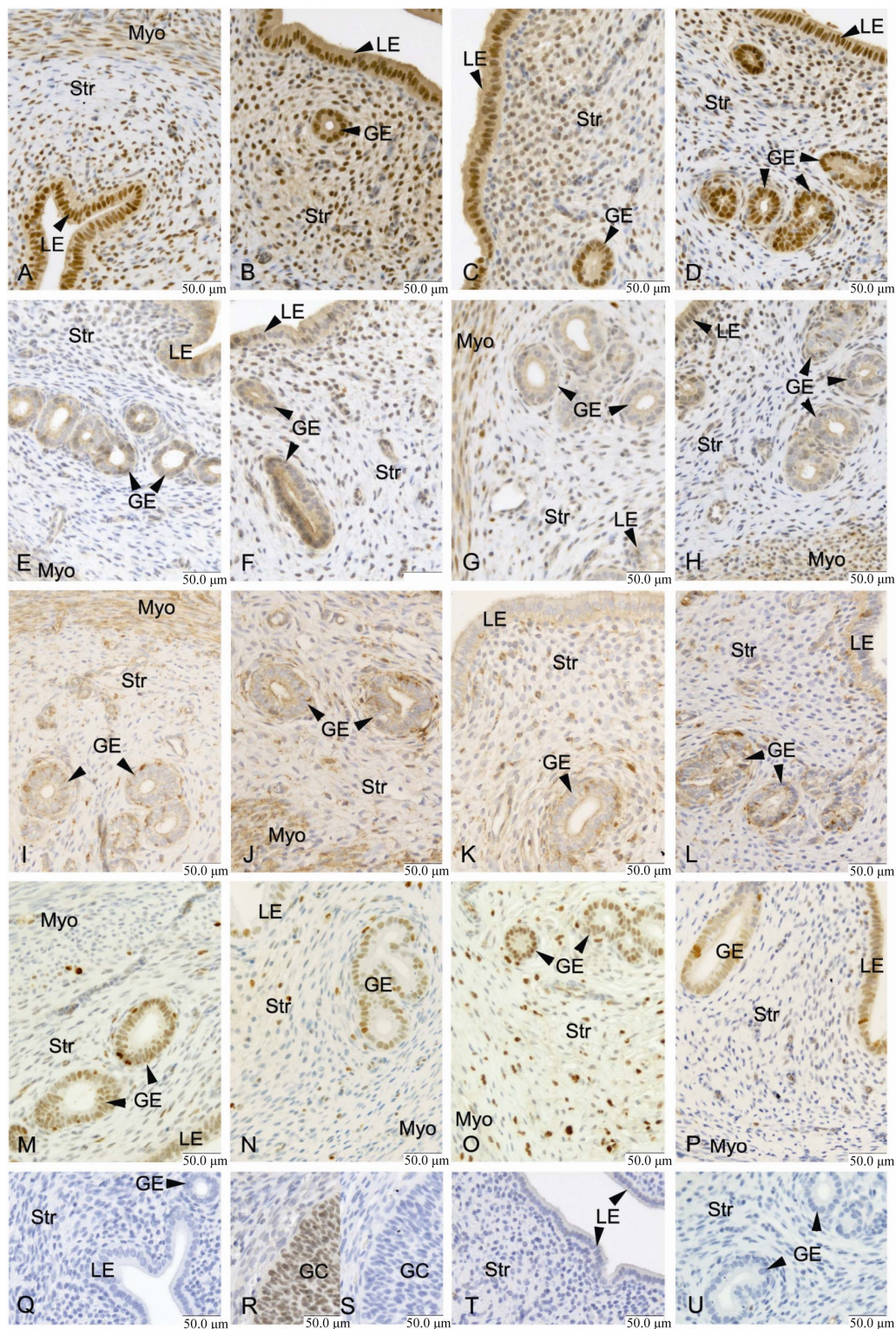


Figure 3. Representative images from immunohistochemistry staining of ER α (A)-(D), ER β (E)-(H), GPER (I)-(L), Ki-67 (M)-(P) and control slides (Q)-(U). LE = luminal epithelium, GE = glandular epithelium, Str = stroma, Myo = myometrium and GC = granulosa cells. The bottom panel shows a negative control for ER α IHC in rat uterus (Q), a positive control for ER β in rat ovary (R), a negative control for ER β in rat ovary (S), and negative controls for GPER (T) and Ki-67 (U) in rat uterus. The images from the different treatment groups are placed in columns for image (A)-(P); OvxC (Column 1), E2 (Column 2), PPT (Column 3) and DPN (Column 4). Magnification $\times 400$, the bar represents 50 μ m.

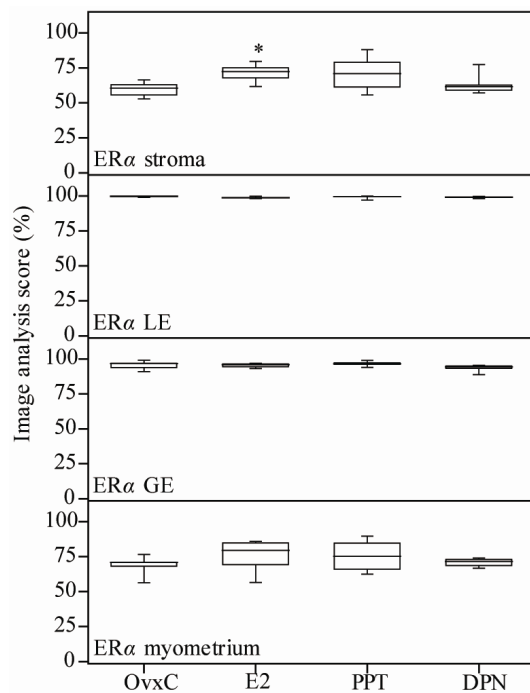


Figure 4. Image analysis scores of ER α immunostaining. OvxC = ovariectomized controls; E2, PPT and DPN treated rats; n = 8 in all groups but the DPN where n = 6. Bars with an asterisk show significant differences to the control group (OvxC) ($p < 0.05$).

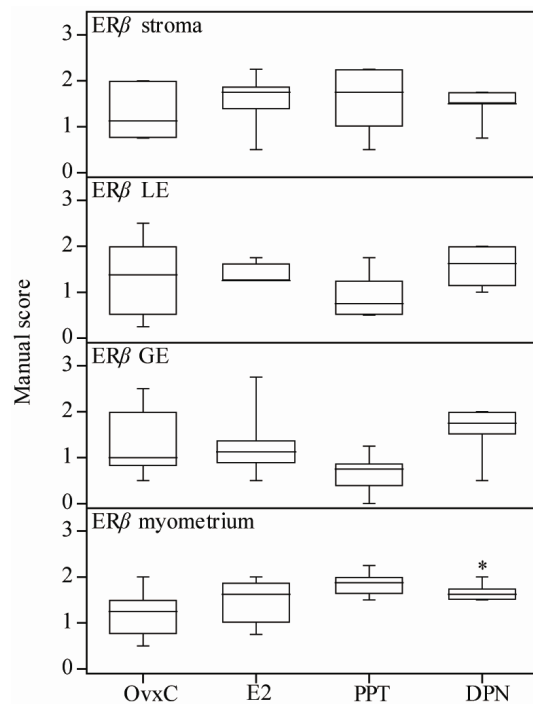


Figure 5. Manual scores of ER β immunostaining. OvxC = ovariectomized controls; E2, PPT and DPN treated rats; n = 8 in all groups but the DPN where n = 6. Bars with an asterisk show significant differences to the control group (OvxC) ($p < 0.05$).

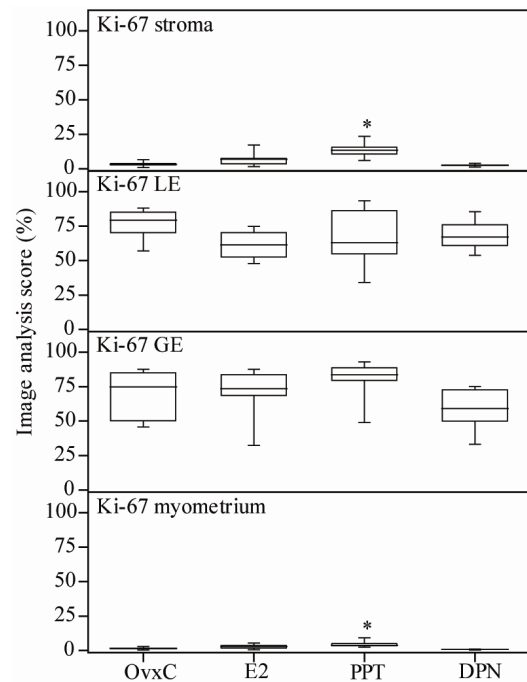


Figure 6. Image analysis scores of the proliferation marker Ki-67 immunostaining. OvxC = ovariectomized controls; E2, PPT and DPN treated rats; n = 8 in all groups but the DPN where n = 6. Bars with an asterisk show significant differences to the control group (OvxC) ($p < 0.05$).

individually.

Selective estrogen receptor agonists were shown to affect PR expression in the uterus differently [34]. PR mRNA levels decreased after DPN treatment. Stromal and myometrial immunostaining of PR increased after E2 and PPT treatment, while no effect was found after DPN treatment [34]. E2 was also found to increase ER α immunostaining in the stroma, thus PR and ER α protein expressions are up regulated in parallel by E2. As opposed to the increased PR immunostaining in myometrium after E2 and PPT treatment, ER β immunostaining was increased only after DPN treatment. Thus, PR and ER β expression might counteract each other, why PR was found increased only when ER β was not, and vice versa.

In conclusion, our study confirms that uterine growth and proliferation is mediated via ER α . ER α activation down regulates ER β mRNA expression in the ovx rat uterus, while ER β binding mediates down regulation of GPER mRNA levels.

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