Studies on estrogen receptor (ER) α and β responses on gene regulation in peripheral blood leukocytes *in vivo* using selective ER agonists

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Abstract

Major reproductive events such as menstruation, ovulation, implantation, and cervical ripening are characterized by an increased number of invading leukocytes in the tissues. Sex steroid hormones, particularly estrogens, play an important role in these dynamic changes in the female reproductive tract. Estrogens have also been implicated in the pathogenesis of many common pathological conditions associated with leukocyte infiltration and immunological dysfunction, such as autoimmune diseases and atherosclerosis. Although the two estrogen receptor (ER) subtypes, ERα and ERβ, have been found in different leukocyte populations in tissues and in peripheral blood, there is still very little known about functional activity and importance of ERs in blood cells. To elucidate the different roles for ERα and ERβ in peripheral blood leukocytes, we used microarray gene expression profiling of rat peripheral blood leukocytes subjected to *in vivo* treatment with estradiol (E_2), the selective ERα agonist 4,4′,4″-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (PPT), and the selective ERβ agonist 2,3-*bis*(4-hydroxyphenyl)-propionitrile (DPN). We report the identification of genes that were commonly regulated by E_2, PPT, and DPN, and genes that were regulated either by the ERα or ERβ agonist. Further confirmatory analyses of the selected regulated genes 12-lipoxygenase, fibulin-1, furin, and calgranulin B are also presented. These results were then compared with those from the uterine tissue of the same animals. Our study demonstrates that peripheral blood leukocytes are responsive to estrogens. E_2 and selective ERα and ERβ agonists regulate a number of genes that may contribute to inflammation and remodeling of the extracellular matrix.

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Introduction

Estrogens play an important role in many normal and pathological conditions associated with leukocyte infiltration. These conditions include dynamic changes in the female reproductive tract as well as autoimmune and cardiovascular diseases.

The majority of autoimmune diseases have higher prevalence in females than in males (Druckmann 2001). The activity of many diseases, such as multiple sclerosis and rheumatoid arthritis, decreases during pregnancy, most profoundly during the third trimester when the plasma estrogen level is highest (Whitacre 2001). Therefore, the sex steroid hormones, primarily estrogens, have been suggested to be responsible for the sex difference in prevalence and presentation of autoimmune diseases. Cardiovascular disorders, such as atherosclerosis, and autoimmune diseases are characterized by leukocyte infiltration and immunological dysfunction. It has been proposed that estrogens modify the course of these disorders by altering leukocyte functions in the tissue (McCrohon *et al.* 1999, Lang 2004).

Leukocyte infiltration is also a distinctive component in the physiological changes observed in the female reproductive tract, where remodeling of the extracellular matrix (ECM) occurs during menstruation, ovulation, implantation, and cervical ripening at parturition (Bokström *et al.* 1997). All these processes are under strict hormonal control, which may indicate that leukocyte function is affected by sex steroid hormones. Cervical ripening is characterized by inflammatory events, such as extravasation of neutrophils and macrophages (Stygar *et al.* 2001, Osman *et al.* 2003). Polymorphonuclear leukocytes and macrophages migrate from blood vessels and accumulate in the cervix uteri before parturition (Stygar *et al.* 2001). The hypothesis of the role of inflammatory cells in cervical ripening (Liggins 1981) has been supported by studies showing that cervical collagenases at parturition originate not only from resident fibroblasts of the cervical stroma but also from leukocytes invading the cervix (Osmers *et al.* 1992). In a previous study, we could show that the matrix metalloproteinase (MMP)-9 is present in the invading leukocytes of the human cervix (Stygar *et al.* 2002). MMPs are a large family of endopeptidases, which are

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responsible for the tissue remodeling and degradation of the ECM including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycan (Verma & Hansch 2007).

Estrogens and selective estrogen receptor (ER) modulators (SERMs) achieve their biological effect through binding to ERs (McDonnell 2006). The two ERs, ERα and ERβ, operate as inducible transcription factors. The ERs are also involved in the recruitment of co-regulator proteins and other protein–protein interactions, which can modify target gene transcription (McKenna *et al.* 1999, Diel 2002). ERα and ERβ are highly homologous in their DNA-binding domains and recognize the same estrogen-responsive element (ERE) on DNA. On the other hand, there is only about 60% homology in the ligand-binding domain, which leaves the possibility that certain ligands can be highly specific to only one subtype of ER (Enmark *et al.* 1997). ERα and ERβ are differentially distributed in tissues and have overlapping, yet different, transcriptional effects. ERα more effectively activates a classical ERE than does ERβ, when stimulated with estradiol (E_2; An *et al.* 1999). In contrast, in response to SERMs, ERβ is more effective at activating transcription via the activating protein (AP)-1 element when compared with ERα (Paech *et al.* 1997).

Several recent studies have aimed at elucidating the effects of selective ER ligands on immune responses. The beneficial effects, particularly of ERα ligands on suppression of experimental autoimmune encephalomyelitis have been shown (Elloso *et al.* 2005). E_2 has been shown to decrease neutrophil infiltration in the lung following trauma hemorrhage in mice (Hildebrand *et al.* 2006), and a selective ERβ ligand (ERB-041) was shown to have potent anti-inflammatory activity in a rat model of arthritis (Follettie *et al.* 2006). The effects of E_2, PPT, and DPN on estrogen-mediated immunomodulation were investigated in ovariectomized (ovx) mice, PPT but not DPN showed effects on thymus (Li & McMurray 2006). Both ER subtypes have been found in different leukocyte populations in human tissues and in peripheral blood (Stygar *et al.* 2001, 2006, Molero *et al.* 2002). Still, the functional activity and importance of ERs in blood cells are principally unknown. To investigate the functional roles for ERα and ERβ in peripheral blood leukocytes, we used microarray gene expression profiling of rat peripheral blood leukocytes subjected to *in vivo* treatment with E_2, the selective ERα agonist 4,4′,4″-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (PPT), and the selective ERβ agonist 2,3-*bis*(4-hydroxyphenyl)-propionitrile (DPN; Harrington *et al.* 2003). We report the identification of genes that were commonly regulated by E_2 and the selective agonists, and genes that were regulated either by the ERα or ERβ agonist. Further confirmatory analyses of the selected regulated genes 12-lipoxygenase (*12-lox*), *fibulin-1*, *furin*, and *calgranulin B* are also presented.

Materials and Methods

Animals and treatments

Thirty-two 8-week-old female Sprague–Dawley rats were obtained from Scanbur BK AB (Sollentuna, Sweden) and housed for 14 days under standard conditions. The animals were housed four per cage with unlimited access to food (standard pellet diet) and water. The animal room was maintained at 21–23 °C with a 12-h light period. On day 0, the animals were ovx. Groups of eight animals (~250 g bw) were s.c. administered 300 μl of either 5·0 μg E_2 or 1·25 mg PPT or 3·125 mg DPN or vehicle on day 14. The animals were killed 18 h after treatment. The doses of PPT and DPN were chosen by comparing data on the activity of the agonists in mouse and rat uterus as reported before (Harris *et al.* 2002, Frasor *et al.* 2003, Lee *et al.* 2005). During the preparation of this article, a paper describing the effects by E_2, PPT, and DPN on the immune functions in mice was published. In that paper, the authors specify doses in the same range as ours (Li & McMurray 2006). The study was approved by the Northern Stockholm Ethical Committee on Animal Care.

Hormones

17β-E_2 was purchased from Sigma Co., 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 <2% in the injections. PPT and DPN were bought from Tocris Cookson, via Bio Nuclear, Bromma, Sweden. These substances were dissolved in DMSO and then diluted with PBS, until the proper concentration and the vehicle was DMSO:PBS 50:50. The control group (OvxC) was injected with the vehicle.

RNA isolation

Blood was collected from *vena cava* in PAXgene Blood RNA tubes (PreAnalytix, Hombrechtikon, Switzerland). A volume of 2·5 ml of blood was collected in each tube and two tubes were obtained from each animal. Prior to RNA isolation, the blood samples were incubated in the tubes for 24 h at room temperature (RT) to achieve complete lysis. Total RNA was purified according to the manufacturer's protocol. The quality and concentration of the RNA were assessed with Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and Nanodrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Five RNA samples of highest quality were selected from each treatment group for microarray gene expression analysis.

The uterine tissue was placed in RNA stabilization solution (RNAlater, Ambion, Austin, TX, USA) immediately after collection and stored at −20 °C. Total RNA from 20 mg uterine tissue from each animal was purified with the RNAeasy kit (Qiagen) according to a procedure

recommended by manufacturer for RNA isolation from fibrous tissues.

Microarray slides

We used spotted 70-mer oligonucleotide microarrays with 27 744 probes from the Operon AROS collection printed in duplicate. The slides were produced by the Swegene DNA Microarray Resource Center, Department of Oncology, Lund University.

cDNA synthesis, labeling, and hybridization

cDNA was synthesized from 20 μg RNA from each sample and reference, and labeled with Cy3 and Cy5 using the CyScribe First-Strand cDNA labeling kit (Amersham Biosciences). RT was primed with anchored oligo(dT) and random primers and catalyzed by CyScript reverse transcriptase in the presence of Cy3 or Cy5 dye-labeled nucleotide dUTP. After RT, the remaining RNA templates were degraded by alkaline hydrolysis treatment using NaOH followed by neutralization with HEPES. Labeled cDNA was purified with QIAquick PCR purification kit (Qiagen). Dye incorporation was estimated using NanoDrop 1000 spectrophotometer. Comparisons were performed on five biological replicates in each treatment group, with three samples in one dye set and two in the opposite, i.e. a dye-swap was done for all treatments. A common reference (vehicle control) was used in all hybridizations.

Microarray slides were incubated with 60 μl pre-hybridization mixture in a humidified chamber in a water bath at 42 °C for 45 min. The pre-hybridization mixture contained 15 μl 20× SSC, 6 μl 50× Denhardt's solution, 1·2 μl tRNA (1 μg/μl), 3 μl 10% SDS, 4·8 μl ddH_2O, and 30 μl formamide. After pre-hybridization, each slide was washed in a Falcon tube four times with 50 ml ddH_2O and dried by centrifugation for 10–15 s in Microarray High-Speed centrifuge MHC220V (TeleChem International, Sunnyvale, CA, USA). The blocking reagents poly d(A) (Amersham Biosciences) and yeast tRNA (Sigma) were added to the cDNA samples in order to reduce non-specific hybridization. Hybridization was performed in a Hybridization Cassette ArrayIt (TeleChem) water bath at 42 °C for 18 h. The hybridization solution contained 20 μg labeled cDNA, 15 μl formamide, 7·5 μl 4× hybridization buffer, 20 μg yeast tRNA, 1 μg poly d(A), and 5 μl ddH_2O. Prior to scanning, the slides were washed with 1×, 0·2×, and 0·1× SSC and dried by centrifugation for 10–15 s.

Scanning and image analysis

Slides were scanned twice at 10 μm resolution with decreasing pmt voltages using a Scanarray Express HT scanner (Perkin–Elmer, Zaventem, Belgium). Image analysis was performed using a SpotReader (Niles Scientific, Portola Valley, CA, USA). Data files were processed using R (R development core team, 2004, http://www.r-project.org) and packages from Bioconductor (www.bioconductor.org). Background was estimated from a local spot background using a 3×3 window minimum and shifted in a way that < 1% of the spots on each array had negative background subtracted intensities.

Data from each array were normalized using a non-stratified loess fit of log ratios on average intensities (Yang *et al.* 2002). Data points from spots near saturation were excluded from the loess fit. Following normalization, data from high pmt voltage scans were combined with data from lower pmt voltage scans by replacing data of probes near saturation in any of the high pmt voltage scans with the corresponding data from the lower pmt voltage datasets. Contrasts between samples of interest and spread measures were calculated using Limma software (http://bioinf.wehi.edu.au/limma; Smyth 2004) on a reduced dataset where probes with less than four spots above an average logged intensity of 9 in all three treatments had been removed. Probes were ordered by the false discovery rate (FDR) reported by Limma when using method FDR as correction for multiple testing (Benjamini & Hochberg 1995).

The gene ontology functional and enrichment analyses

Gene ontology functional and term enrichment analyses were carried out using the DAVID tool (http://david.abcc.ncifcrf.gov/) and by applying EASE statistics for the enrichment analysis (Hosack *et al.* 2003). First, for each probe on the array, the Ensembl IDs provided by the array probe manufacturer were converted to Entrez Gene IDs using the BioMart tool (http://www.biomart.org). Next, for each set of genes (corresponding to either up- or down-regulated genes by the E_2, PPT, or DPN treatments, i.e. six lists in total), the co-occurrence of gene ontology functional terms in the annotations were compared with the expected frequencies derived by using a background list corresponding to all probes printed on the array. The results are presented in supplementary Tables 1 and 2 (see supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol194/issue2). A term was considered significantly enriched if the P value was < 0·05 and if at least two genes mapped to the term.

ArrayExpress

The entire microarray dataset has been deposited into the ArrayExpress microarray data repository using accession numbers A-MEXP-743 (70-mer microarray platform) and A-TABM-269 (gene expression dataset).

Reverse Transcription (RT)

Total RNA (2 μg) from each leukocyte and uterus sample were reverse transcribed at 37 °C for 60 min in a final volume of 20 μl with a reaction mixture (Qiagen GmbH) containing

1× RT buffer, dNTP mix (0·5 mM each dNTP), 600 ng random primers (Invitrogen), 10 units RNase inhibitor (Superase-In, Ambion), and 4U of Omniscript reverse transcriptase (Qiagen).

Real-time PCR for 12-lox, fibulin-1, furin, and calgranulin B

Real-time PCR was performed in a DNA Engine Opticon 2 System (MJ Research, Waltham, MA, USA). For PCR, the cDNAs corresponding to 100 ng RNA were added to 10 µl Quantitect SYBR® Green PCR mix (Qiagen) containing HotStarTaq DNA polymerase, PCR buffer, dNTP mixture, and 0·3 µM of each oligonucleotide primer in a final volume of 20 µl. The reactions were performed in opaque white 0·2 ml low-profile strip tubes sealed with optical flat caps (TLS-0851, TCS-0803, MJ Research). After initial incubation for 15 min at 95 °C, the samples were subjected to 44 cycles of 10 s at 94 °C, 15 s at 57 °C, and 20 s at 72 °C with a final extension step at 72 °C for 5 min. All reactions were performed in duplicates. The amount of PCR products for *12-lox, fibulin-1, furin,* and *calgranulin B* in peripheral blood and uterus samples increased linearly up to 25–28 cycles. The purity of PCR products was confirmed by a melting curve analysis in all experiments (data not shown). Oligonucleotide primers for *12-lox, fibulin-1, fibulin-1C, fibulin-1D, furin, calgranulin B* and *HPRT* are listed in Table 1. All primers were designed to span an intron/exon boundary or to flank an intron; thus, amplification of contaminating DNA was eliminated. Each PCR assay included a negative control containing RNA sample without RT.

Quantification of mRNA

To standardize the quantification method, hypoxanthine guanine phosphoribosyl transferase (*HPRT*) was selected out of several tested housekeeping genes as an invariable internal control. The control gene, 12-lox, fibulin-1, furin, and calgranulin B mRNAs were amplified under the same conditions. The PCR amplification rate and the cycle threshold (Ct) values were related to a standard curve using Opticon Monitor 2.0 software (MJ Research). The values of relative expression of genes of interest were normalized against the HPRT product.

Protein extraction

Peripheral blood leukocytes and uterus were stored at −20 °C in RNAlater stabilization solution (Ambion). Leukocytes (3–4×10^6) and uterus tissue (20–40 µg) were disrupted, respectively, in 300 and 400 µl ice-cold cell lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 1% Triton-X, and 5 mM EDTA, as well as a protease inhibitor (Complete Mini, Roche). Protein concentrations were measured by Lowry protein assay with BSA as a standard (Lowry *et al.* 1951).

Western blot

Leukocyte samples from two ovx rats and from two ovx- and 17β-E_2-treated rats from a previous experiment were available for western blot. The leukocyte samples from the animals in the present study were not obtainable for protein preparation, since all material was used for RNA extraction for the microarray experiment, and later also for PCR. However, protein samples from all the uteri of the animals in the present experiment were used. Protein extract (10 µg) from each sample was resolved on 7·5% polyacrylamide gels for 12-lox, whereas 60 µg samples were run on 15% polyacrylamide gels for calgranulin B, in Mini-Protean II cell (Bio-Rad Laboratories) using Tris–glycine buffers. Proteins were transferred from the gels to polyvinylidene fluoride (PVDF) membranes (Amersham) by electroblotting. Molecular weight marker was purchased from Bio-Rad (Precision Plus protein

Table 1 Oligonucleotide primers used for real-time PCR

Gene	Accession no. or reference	Primer	Position
12-Lipoxygenase	NM_031010	Forward GAAGCTGCTACGACCCTGTC	Exon 1, bp 114–133
		Reverse GGCGTCATCCGTGAGATAAT	Exon 2, bp 231–212, product 118 bp
Fibulin-1	XM_243637	Forward CAAGGAGTGCAGGATGGTC	Exon 3–4, bp 304–322
		Reverse TGGTCTCGAGGCTACTGTTG	Exon 4, bp 440–421, product 137 bp
Fibulin-1C	Haendler *et al.* (2004)	Forward CTGCAGACACCCGCTGTG	Exon 14, bp 1694–1712
		Reverse AGCGGTGATGGCCAGCTG	Exon 15, bp 1875–1858, product 182 bp
Fibulin-1D	XM_243637	Forward GGCTGACATCATCTTCGACA	Exon 16, bp 2023–2042
		Reverse AGCTTCAGGACGGCATAAAA	Exon 17, bp 2163–2144, product 141 bp
Furin	NM_019331	Forward TAGCTGCCAGACCACATGAC	Exon 13–14, bp 1990–2009
		Reverse GGTCAGCGTCCCATAGTTGT	Exon 14–15, 2135–2116, product 146 bp
Calgranulin B	NM_053587	Forward GCTCCTTAGCTTTGAGCAAGA	Exon 1–2, bp 9–29
		Reverse TTTCTTTGAATTCCGCCTTG	Exon 2, bp 152–133, product 144 bp
HPRT	NM_012583	Forward CTCATGGACTGATTATGGACAGGAC	Exon 2–3, bp 179–203
		Reverse GCAGGTCAGCAAAGAACTTATAGCC	Exon 3, bp 301–277, product 123 bp

standard, Bio-Rad Laboratories). All uterine samples from the experiment were analyzed by western blot, run two from each group in four consecutive gels.

After blocking in 5% non-fat milk (NFM, Bio-Rad Laboratories) in TBS–Tween (0·1%) for 60 min at room temperature, the membranes were incubated overnight at 4 °C with primary antibodies. The dilution of the primary antibodies was 1:1500 2·5% NFM in TBS–T for the 12-lox polyclonal antiserum (rabbit anti-mouse, Cat. No. 160304, Cayman Chemical, Ann Arbor, MI, USA) and 1:100 in 2·5% NFM in TBS–T for the calgranulin B polyclonal antibody (goat anti-mouse, Cat. No. sc-8115, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed twice for 5 min in TBS–T and incubated for 60 min at RT with AP-conjugated anti-rabbit secondary antibody (Sigma sc-3687) diluted 1:15 000 in 2·5% NFM in TBS–T or with AP-conjugated anti-goat secondary antibody (Cat. No. sc-2310, Santa Cruz Biotechnology) diluted 1:500 in 2·5% NFM in TBS–T. This was followed by washing in TBS–T and developing the membranes in NBT/BCIP (Sigma). All incubations were performed on a shaking platform.

The bands from the 12-lox blots were captured and analyzed using the Gel Doc 2000 Gel Documentation System (Bio-Rad Laboratories).

Statistical analyses

Data from the relative quantification of real-time PCR products and western blot were analyzed with ANOVA on ranks (Kruskal–Wallis test). Significance levels were calculated using Dunn's test. Differences at $P<0{\cdot}05$ were considered significant.

Results

Uterine weights

The uterine weights of the rats were measured to determine the estrogenic response from the treatment. The uterine weights (in g ± S.E.M.) of the animals in the E_2 and PPT groups (0·169 ± 0·009 and 0·205 ± 0·009 respectively) were significantly increased when compared with the OvxC group (0·120 ± 0·007), while the DPN group was unaffected (0·120 ± 0·005). This confirms that ERα mediates the effect on uterine weight.

Genes regulated by E_2, PPT, and DPN

The identification of genes reproducibly regulated by the treatments is based on the low FDR criterion. We considered the regulated genes to be those with an absolute *t*-value (a ratio of Log2-expression level to its standard error) higher than or equal to 3 (Fig. 1). Under these conditions, we found 54 up-regulated and 96 down-regulated genes. As shown in the Venn diagrams, most of the induced or suppressed genes identified by microarray analysis were unique in each

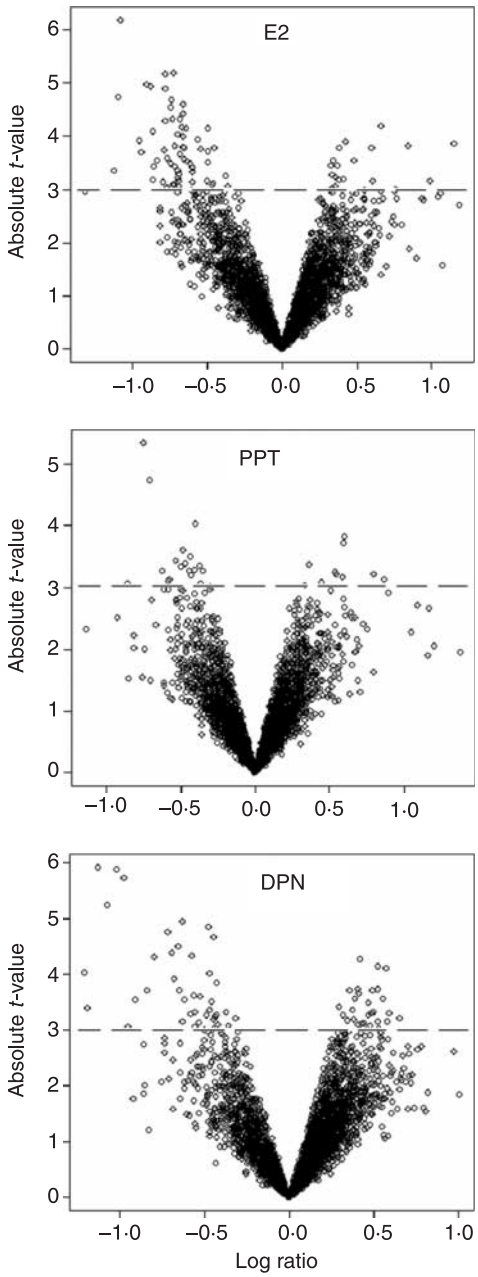

Figure 1 Scatter plots of an absolute *t*-value (a ratio of Log2-expression level to its standard error), when testing for difference between each treatment and control, plotted versus mean log difference between treatment and control. Genes with statistically significant differential expression according to moderated *t*-statistics lie above a horizontal threshold line.

treatment group. Only a few genes were commonly regulated by two or more treatments (Fig. 2). We observed more negatively regulated than induced genes in all treatment groups. The selective ERβ agonist DPN produced considerably greater transcriptional response in leukocytes when compared with the selective ERα agonist PPT (Fig. 2).

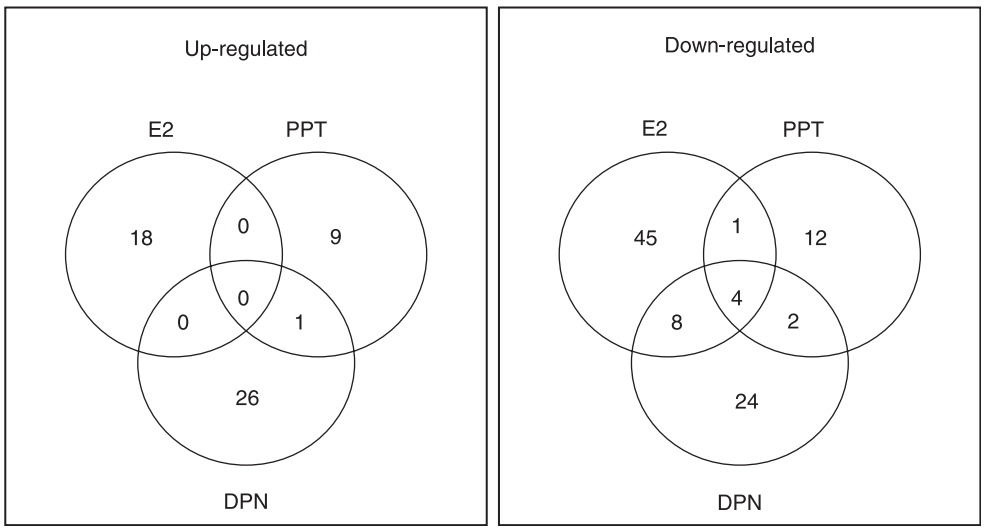

Figure 2 Number of common and unique up- and down-regulated probes with an absolute *t*-value more than or equal to 3 when testing for difference between each treatment and control.

Tables 2–4 list genes that were regulated by each treatment and had an absolute *t*-value more or equal to 3. Among regulated genes identified by microarray, we selected a few genes that may be involved in the remodeling of ECM. Transcriptional profiles of the selected genes were also determined by real-time PCR. These genes were arachidonate *12-lox*, *fibulin-1*, *furin*, and *calgranulin B* (highlighted in bold in Tables 2–4).

Functional categories and enrichment analyses

Studies on the functional categories of the regulated genes revealed that these analyses were possible to do on down-regulated genes for all treatments but in the case of up-regulated genes only after DPN treatment due to the limited number of genes that are up-regulated by the E_2 and PPT treatments. What we find is that the majority of down-regulated genes, as well as the shared down-regulated genes, relate to 'cellular metabolism' and 'intracellular organelle/intracellular membrane-bound organelle/cytoplasm'. Comparing up- and down-regulated genes after DPN treatment shows that genes related to metabolism are regulated both ways, while 'cell death' is among the down-regulated genes and 'immune response' and 'defense response' are among the up-regulated genes. In addition, genes related to 'metal ion binding' and 'cation binding' are down-regulated, while those associated with 'RNA binding' are up-regulated. Thus, there are both functional similarities and differences between up- and down-regulated genes after DPN treatment. For the functional categories of regulated genes on term basis, please see supplementary Table 1a–d.

The enrichment analysis is carried out for all gene ontology branches ('molecular function', 'biological processes', and 'cellular component') and KEGG pathways. The results of the analyses are reported in supplementary Tables 2a and b, when enriched terms were found ($P<0{\cdot}1$). Note that the Tables also contain terms that do not reach the statistical significance, but are close to ($0{\cdot}1>P>0{\cdot}05$); these are written in italics. The general conclusion from this analysis is that the gene lists are too short for (gene ontology) term enrichment analysis. An exception to this is the group of genes down-regulated by E_2 treatment, for which multiple (mainly overlapping) terms are found (see supplementary Table 2 for details at http://joe.endocrinology-journals.org/content/vol194/issue2). Down-regulation after E_2 treatment relates to 50% or more to 'binding' and 'cellular physiological process' ($P<0{\cdot}05$). The most significantly affected functions were 'responses to oxidative stress' and 'oxygen and reactive oxygen species metabolism' ($P<0{\cdot}001$) together with 'negative regulation of cellular physiological process' ($P<0{\cdot}01$) and 'cellular metabolism' ($P=0{\cdot}01$). PPT showed no significantly enriched terms among the down-regulated genes, but the terms closest to significance ($0=0{\cdot}054$ and $0{\cdot}059$ respectively) were 'endosome' and 'intracellular transport'. Looking at DPN treatment, the significantly enriched terms for the down-regulated genes were those related to the terms 'Golgi network', 'intracellular', and 'transport vesicle'. The up-regulated genes were associated with the terms 'ribosome', 'cytosol', 'non-membrane bound organelles', and 'immune response'.

The enrichment analysis clearly shows that E_2 primarily down-regulate genes associated with physiological processes, metabolism, and binding, while DPN down-regulate genes associated with the Golgi network and intracellular transport (supplementary Table 2a at http://joe.endocrinology-journals.org/content/vol194/issue2).

In addition, we find that up- and down-regulated genes after DPN treatment affect different functions; intracellular functions are down-regulated, while genes related to ribosomal functions and immune responses are up-regulated (supplementary Table 2b http://joe.endocrinology-journals.org/content/vol194/issue2).

Table 2 Subset of genes regulated by estradiol in peripheral blood leukocytes

Ensembl gene_ID	Average log ratio, M	**Description** (Accession no.)	**Molecular function/biological process**
ENSRNOG00000003875	1·15	Predicted: RIKEN cDNA 9530014D17 (XP_229106)	Inositol or phosphatidylinositol phosphatase/catalytic activity
ENSRNOG00000003139	0·99	Structural maintenance of chromosome 1-like 1 protein (Q9Z1M9)	Nucleotide binding, chromatin binding/cell cycle, mitosis
ENSRNOG00000026963	0·84	Tumor rejection antigen gp96 (NP_001012197)	ATP binding, unfolded protein binding/-protein folding
ENSRNOG00000017469	0·66	Annexin A1 (phospholipase A2 inhibitory protein) (P07150)	Calcium-dependent phospholipase A2 inhibitor/insulin secretion
ENSRNOG00000010218	0·61	Eukaryotic translation-initiation factor 5 (Q07205)	Translation initiation factor activity/protein biosynthesis
ENSRNOG00000014228	0·60	Hemoglobin β chain, minor-form (P11517)	
ENSRNOG00000028015	0·48	Platelet factor 4 precursor (PF-4; CXCL4; P06765)	Chemokine activity, heparin binding/immune response
ENSRNOG00000024888	0·42		
ENSRNOG00000019460	0·41	Signal sequence receptor 4 (NM_017199)	
ENSRNOG00000028746	0·39	Glutathione transferase omega 1 (Q9Z339)	Glutathione transferase/metabolism, ascorbic acid biosynthesis
ENSRNOG00000027712	0·38		
ENSRNOG00000025967	0·37	40S ribosomal protein S28 (P25112)	
ENSRNOG00000020169	0·36	Regulator of G-protein signaling 10 (RGS10; P49806)	GTPase activator/G-protein coupled receptor signaling pathway
ENSRNOG00000025204	0·35		
ENSRNOG00000021808	0·34	Synaptic glycoprotein SC2 (Q64232)	Integral to membrane
ENSRNOG00000021724	0·33		
ENSRNOG00000024812	0·33	60S Ribosomal protein L39 (P02404)	
ENSRNOG00000000261	0·33		
ENSRNOG00000017983	−0·36	Ubiquitin-associated domain containing 1 (NM_001007742)	
ENSRNOG00000015385	−0·37	Predicted: protein kinase BRPK (XP_216565)	Protein serine–threonine kinase activity/-protein phosphorylation
ENSRNOG00000003594	−0·45		
ENSRNOG00000018998	−0·46	Predicted: K cell-specific antigen KLIP1 (NP_001007802)	
ENSRNOG00000020667	−0·47	Scotin (NM_001006989)	Activator of apoptosis?
ENSRNOG00000020526	−0·49	Predicted: RIKEN cDNA 9130404D08 (XP_214306)	
ENSRNOG00000011633	−0·50	α-parvin (Actopaxin; Q9HB97)	Catalytic activity
ENSRNOG00000021698	−0·50	M-phase inducer phosphatase 2 CDC25B (P48966)	
ENSRNOG00000018316	−0·54		
ENSRNOG00000023185	−0·56		
ENSRNOG00000019183	−0·60	**Arachidonate 12-lipoxygenase, leukocyte-type** (Q02759)	Lipoxygenase activity/arachidonic acid metabolism
ENSRNOG00000012557	−0·60	Galectin-5 (RL-18; P47967)	Sugar binding
ENSRNOG00000008364	−0·60	Catalase (P04762)	Catalase activity/metal ion binding, electron transport
ENSRNOG00000008628	−0·61	Islet cell autoantigen 1 (ICAp69; Q63054)	Molecular function unknown
ENSRNOG00000021170	−0·64		
ENSRNOG00000017854	−0·64	Mitochondrial uncoupling protein 2 (P56500)	Binding/transport, mitochondrial transport
ENSRNOG00000018000	−0·65	Predicted: Ran-binding protein 10 (XP_341677)	Catalytic activity
ENSRNOG00000006770	−0·66		
ENSRNOG00000016390	−0·66	Predicted: translation elongation factor 1 epsilon 1 (XP_214451)	
ENSRNOG00000025534	−0·66	Erythroid transcription factor (GATA-1; P43429)	Metal ion binding/regulation of transcription, DNA-dependent
ENSRNOG00000026119	−0·66	14-3-3 protein η (P68511)	Protein domain-specific binding
ENSRNOG00000027368	−0·66		
ENSRNOG00000023168	−0·67		

(continued)

Table 2 *Continued*

	Average log ratio, M	**Description** (Accession no.)	**Molecular function/biological process**
ENSRNOG00000016150	−0·68	Predicted: IRFD2 (XP_217254)	
ENSRNOG00000018325	−0·69		Integral to membrane
ENSRNOG00000014179	−0·69	40S ribosomal protein S2 (P27952)	Structural constituent of ribosome/protein biosynthesis
ENSRNOG00000007921	−0·69	Heterogeneous nuclear ribonucleoprotein M (Q62826)	RNA binding
ENSRNOG00000025323	−0·70		
ENSRNOG00000004169	−0·70	Predicted: fizzy-related protein (XP_243390)	Catalytic activity
ENSRNOG00000010037	−0·70	Predicted: erythrocyte membrane protein band 4·1 (XP_232771)	Actin binding/catalytic activity, cytoskeleton organization
ENSRNOG00000017671	−0·72	Ras GTPase-activating protein 3 (Q9QYJ2)	GTPase activator activity/catalytic activity
ENSRNOG00000009640	−0·72	Proteasome inhibitor PI31 subunit (Q5XIU5)	Protein complex
ENSRNOG00000025023	−0·72	Signal transducer and activator of transcription 6, STAT6 (O70429)	Transcription factor activity/regulation of transcription
ENSRNOG00000007971	−0·72	WW domain-binding protein 2 (Q8R478)	
ENSRNOG00000020235	−0·73	Heterogeneous nuclear ribonucleoprotein L (NP_116008)	Nucleic acid binding
ENSRNOG00000011489	−0·73	Predicted: RIKEN cDNA C030046I01 (XP_343169)	Nucleic acid binding
ENSRNOG00000018053	−0·74	Predicted: ferrochelatase (XP_341623)	Ferrochelatase activity/heme biosynthesis
ENSRNOG00000022927	−0·74		
ENSRNOG00000019349	−0·74	Splicing factor 3a, subunit 2 (NP_001011986)	Nucleic acid binding, zinc ion binding
ENSRNOG00000018396	−0·76	Pre-mRNA splicing factor 18 (Q9JKB8)	Potassium channel inhibitor activity/RNA splicing
ENSRNOG00000020102	−0·77	Sirtuin 2 (silent mating type information regulation 2; NP_001008369)	DNA binding, chromatin silencing/regulation of transcription
ENSRNOG00000005480	−0·78	DNA-binding protein A (Q62764)	Single-stranded DNA binding/regulation of transcription
ENSRNOG00000019721	−0·78	Predicted: yippee-like 3 (XP_215057)	
ENSRNOG00000012206	−0·78	Predicted: Rab6 interacting protein 1 (XP_219270)	Nucleic acid binding/catalytic activity
ENSRNOG00000020363	−0·78	Predicted: mediator of RNA polymerase II subunit 9 (XP_214950)	Catalytic activity
ENSRNOG00000015753	−0·78	Epsin-1 (EPS-15 interacting protein 1; O88339)	Lipid binding/endocytosis
ENSRNOG00000015762	−0·79	Ankyrin repeat and BTB (POZ) domain containing 1 (NP_001005902)	Protein binding
ENSRNOG00000016185	−0·83		
ENSRNOG00000020994	−0·85		Binding/transport
ENSRNOG00000017871	−0·86	Predicted: to Sidt2 protein (XP_236205)	Catalytic activity
ENSRNOG00000009323	−0·86		
ENSRNOG00000007946	−0·88	Apoptosis regulator Bcl-X (P53563)	Caspase inhibitor activity/anti-apoptosis
ENSRNOG00000011352	−0·91	**Furin precursor** (P23377)	Calcium ion binding, proteolysis and peptidolysis/catalytic activity
ENSRNOG00000020951	−0·94	Band 3 anion transport protein (P23562)	Inorganic anion exchanger activity/anion transport
ENSRNOG00000018503	−0·96	Bcl-x short form (Q548R7)	Regulation of apoptosis
ENSRNOG00000021248	−1·08	M-phase inducer phosphatase 2 (P48966)	Protein tyrosine phosphatase activity/cell division
ENSRNOG00000014137	−1·10	Predicted: **fibulin-1 precursor** (XP_243637)	Calcium ion binding/catalytic activity
ENSRNOG00000016984	−1·12	Predicted: hypothetical protein D2Ertd391e (XP_342466)	

Table 3 Subset of genes regulated by 4,4′,4″-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol in peripheral blood leukocytes

	Average log ratio, M	**Description** (Accession no.)	**Molecular function/biological process**
Ensembl gene_ID			
ENSRNOG00000009263	0·87	Putative ISG12(a) protein (NP_981955)	Response to pest, pathogen, or parasite
ENSRNOG00000023887	0·80		
ENSRNOG00000027225	0·61	G6f protein (NP_001003691)	
ENSRNOG00000022940	0·60		
ENSRNOG00000010906	0·59	Small inducible cytokine A5 precursor (CCL5) RANTES (P50231)	Chemokine activity/chemotaxis, inflammatory response
ENSRNOG00000024605	0·54		
ENSRNOG00000021216	0·54	Predicted: peroxisomal biogenesis factor 11b (NM_001025684)	
ENSRNOG00000025440	0·45		
ENSRNOG00000023720	0·37	Predicted: neurotrimin precursor (GP65; Q62718)	Protein binding/cell adhesion
ENSRNOG00000004335	0·33		
ENSRNOG00000009094	−0·30	Diphosphoinositol polyphosphate phosphohydrolase 2 (NM_053598)	Magnesium ion binding/intracellular signaling cascade
ENSRNOG00000023647	−0·35	Fructose-bisphosphate aldolase A (P05065)	Lyase activity/glycolysis
ENSRNOG00000011633	−0·37	α-parvin (Actopaxin; Q9HB97)	Catalytic activity
ENSRNOG00000027223	−0·37		
ENSRNOG00000003220	−0·40	Histone H3.3 (P84245)	DNA binding/chromosome organization and biogenesis
ENSRNOG00000018735	−0·40	H-2 class II histocompatibility antigen (CD74 antigen; P10247)	Cytokine binding, prostaglandin biosynthesis/immune response
ENSRNOG00000009643	−0·43	Predicted: RIKEN cDNA 4121402D02 (XP_239335)	
ENSRNOG00000017667	−0·44	Predicted: mKIAA0863 protein (LOC307236; XM_225732)	
ENSRNOG00000007628	−0·44	Predicted: protein tyrosine phosphatase 4a3 (XP_343260)	Protein tyrosine phosphatase/protein dephosphorylation
ENSRNOG00000028530	−0·47		
ENSRNOG00000025986	−0·48		
ENSRNOG00000012206	−0·50	Predicted: Rab6 interacting protein 1 (XP_219270)	Nucleic acid binding/catalytic activity
ENSRNOG00000017854	−0·53	Mitochondrial uncoupling protein 2 (P56500)	Transporter/mitochondrial transport
ENSRNOG00000011352	−0·57	**Furin precursor** (P23377)	Calcium ion binding, proteolysis and peptidolysis/catalytic activity
ENSRNOG00000009721	−0·58	Olfactory receptor Olr483 (NP_001000683)	Olfactory receptor/G-protein coupled receptor signaling pathway
ENSRNOG00000010266	−0·62	Predicted: RP105 (XP_226731)	
ENSRNOG00000016390	−0·71	Predicted: translation elongation factor 1 epsilon (XP_214451)	
ENSRNOG00000023417	−0·75		
ENSRNOG00000017962	−0·85	Serine/cysteine proteinase inhibitor, clade B6 (NP_954516)	Serine-type endopeptidase inhibitor activity

Detection of 12-lox, furin, calgranulin B, and fibulin-1 in blood and uterus by real-time PCR

To investigate whether genes identified by microarray in peripheral blood leukocytes are regulated in other tissues, we examined the effect of E_2, PPT, and DPN also on their expression in rat uterus by real-time PCR.

The microarray results showed that 12-lox was down-regulated in the E_2 and DPN groups (Tables 2 and 4). This effect was partly confirmed by real-time PCR, where 12-lox expression was found negatively regulated in leukocytes from the DPN group (Fig. 3A). In addition, a tendency to decrease in the 12-lox mRNA levels was found in the two other treatment groups (Fig. 3A). We observed a stronger down-regulated expression pattern of 12-lox in uterus, when compared with leukocytes. The decrease in the uterine 12-lox mRNA levels was most prominent in the E_2 and PPT groups (Fig. 3B), while in leukocytes, the maximum effect was observed in the DPN group (Fig. 3A).

The furin precursor was found to be down-regulated by all treatments (Tables 2–4). Real-time PCR also showed a tendency to down-regulation by all treatments, but the

Table 4 Subset of genes regulated by 2,3-*bis*(4-hydroxyphenyl)-propionitrile in peripheral blood leukocytes

	Average log ratio, M	**Description** (Accession no.)	**Molecular function/biological process**
Ensembl gene_ID			
ENSRNOG00000010203	0·66	Macrophage-inducible C-type lectin member 9 (Q67EQ1)	Sugar binding/immune response
ENSRNOG00000008472	0·59	RNA-binding protein 10 (P70501)	
ENSRNOG00000010906	0·58	Small inducible cytokine A5 precursor (CCL5) RANTES (P50231)	Chemokine activity/chemotaxis, inflammatory response
ENSRNOG00000028235	0·58	Predicted: sepiapterin reductase (XP_216190)	
ENSRNOG00000010306	0·55	Histone H2A.z (P17317)	DNA binding/chromosome organization and biogenesis
ENSRNOG00000008899	0·53		
ENSRNOG00000004737	0·53	MRC OX-45 surface antigen precursor (CD48; P10252)	Mast cell activation/immune response
ENSRNOG00000013548	0·53	Selenoprotein W (P63301)	Selenium binding, oxidoreductase activity/cell redox homeostasis
ENSRNOG00000020983	0·53		
ENSRNOG00000011483	0·50	**Calgranulin B** (P50116)	Calcium ion binding
ENSRNOG00000016507	0·49		
ENSRNOG00000016810	0·49	Stathmin (Phosphoprotein p19; P13668)	Tubulin binding/intracellular signaling cascade
ENSRNOG00000025040	0·48	Guanine nucleotide-binding protein γ 10 (NP_446112)	Signal transducer/G-protein coupled receptor protein signaling
ENSRNOG00000028747	0·47	Ribosomal protein L22 (NM_031104)	
ENSRNOG00000027006	0·47	Heterogeneous nuclear ribonucleoprotein A3 (Q6URK4)	Nucleic acid binding/ribonucleoprotein complex
ENSRNOG00000023159	0·44	40S ribosomal protein S18 (P25232)	
ENSRNOG00000012383	0·44	Predicted: NADH dehydrogenase 1 (NP_001009290)	NADH dehydrogenase activity/mitochondrial electron transport
ENSRNOG00000015455	0·43	Sepiapterin reductase (P18297)	Sepiapterin reductase/metabolism
ENSRNOG00000022396	0·42		
ENSRNOG00000025973	0·41	60S ribosomal protein L44 (L36A; P09896)	Structural constituent of ribosome/protein biosynthesis
ENSRNOG00000023288	0·41		
ENSRNOG00000021906	0·41	60S ribosomal protein L9 (P17077)	
ENSRNOG00000021853	0·39	60S ribosomal protein L35A (P04646)	
ENSRNOG00000006423	0·39	Peptidyl-prolyl cis-trans isomerase A (P10111)	Cyclosporin A binding/protein folding
ENSRNOG00000004214	0·36	60S ribosomal protein L26 (P12749)	Structural constituent of ribosome/protein biosynthesis
ENSRNOG00000024472	0·35		
ENSRNOG00000008551	0·32	40S ribosomal protein S7 (P62083)	Structural constituent of ribosome/protein biosynthesis
ENSRNOG00000003220	−0·32	Histone H3.3 (P84245)	DNA binding/chromosome organization and biogenesis
ENSRNOG00000019534	−0·36	Adapter-related protein complex 2 α 2 subunit (P18484)	Structural molecule, protein complex assembly/protein transport
ENSRNOG00000014743	−0·37	Hydroxyacylglutathione hydrolase (O35952)	Hydroxyacylglutathione hydrolase activity, metal ion binding
ENSRNOG00000018783	−0·42	Predicted: breast carcinoma amplified sequence 2 (XP_215664)	
ENSRNOG00000000701	−0·42	Predicted: nitrogen fixation cluster-like (XP_213811)	
ENSRNOG00000028411	−0·42	PEST-containing nuclear protein (PCNP; Q7TP40)	Cell cycle
ENSRNOG00000021248	−0·44	M-phase inducer phosphatase 2 (P48966)	Protein tyrosine phosphatase activity/cell division
ENSRNOG00000005522	−0·44	Predicted: Sh3yl1 (XP_343046)	Catalytic activity
ENSRNOG00000021848	−0·44		
ENSRNOG00000000167	−0·45		
ENSRNOG00000011648	−0·46	Aquaporin-1 (P29975)	Transporter/membrane transport
ENSRNOG00000009280	−0·47	Makorin, ring finger protein, 1 (NP_001004233)	Nucleic acid binding/protein ubiquitination
ENSRNOG00000007971	−0·47	WW domain-binding protein 2 (Q8R478)	

(continued)

Table 4 *Continued*

	Average log ratio, M	**Description** (Accession no.)	**Molecular function/biological process**
ENSRNOG00000021207	−0·47	Predicted: galectin-12 (XP_219545)	Sugar binding
ENSRNOG00000016390	−0·49	Predicted: translation elongation factor 1 epsilon 1 (XP_214451)	
ENSRNOG00000007136	−0·53	Annexin A7 (NM_130416)	Phospholipid binding/regulation of coagulation
ENSRNOG00000013461	−0·53	RalA-binding protein 1 (NM_032067)	GTPase activator/transport, cell cycle, mitosis
ENSRNOG00000017640	−0·55	Calcium-binding protein P22 (NM_024139)	Calcium ion binding
ENSRNOG00000011352	−0·56	**Furin precursor** (P23377)	Calcium ion binding, proteolysis and peptidolysis/catalytic activity
ENSRNOG00000017854	−0·56	Mitochondrial uncoupling protein 2 (P56500)	Binding/transport, mitochondrial transport
ENSRNOG00000025986	−0·58		
ENSRNOG00000016670	−0·58	Phosphatidylinositol-4-phosphate 5-kinase, II α (NM_053926)	
ENSRNOG00000019113	−0·63	Heterogeneous nuclear ribonucleoprotein K (NM_057141)	Nucleic acid binding
ENSRNOG00000018184	−0·63	Tropomyosin 1 α chain (P04692)	Actin binding/cytoskeleton
ENSRNOG00000009600	−0·64		
ENSRNOG00000019366	−0·65		
ENSRNOG00000018000	−0·68	Predicted: Ran-binding protein 10 (XP_341677)	Catalytic activity
ENSRNOG00000018053	−0·69	Predicted: ferrochelatase (XP_341623)	Ferrochelatase activity/heme biosynthesis
ENSRNOG00000012206	−0·72	Predicted: Rab6 interacting protein 1 (XP_219270)	Nucleic acid binding/catalytic activity
ENSRNOG00000006770	−0·79		
ENSRNOG00000015420	−0·84	Syntaxin-binding protein 1 (P61765)	Vesicle docking during exocytosis/protein transport
ENSRNOG00000020951	−0·90	Band 3 anion transport protein (P23562)	Inorganic anion exchanger activity/anion transport
ENSRNOG00000025235	−0·95	LOC304280 protein (Q5RJK4)	Catalytic activity
ENSRNOG00000019183	−0·98	**Arachidonate 12-lipoxygenase, leukocyte-type** (Q02759)	Lipoxygenase activity/arachidonic acid metabolism
ENSRNOG00000010037	−1·02	Predicted: erythrocyte membrane protein band 4·1 (XP_232771)	Actin binding/catalytic activity, cytoskeleton organization
ENSRNOG00000018241	−1·07		
ENSRNOG00000019183	−1·13	**Arachidonate 12-lipoxygenase, leukocyte-type** (Q02759)	Lipoxygenase activity/arachidonic acid metabolism
ENSRNOG00000022678	−1·19		
ENSRNOG00000018282	−1·21	Guanine deaminase (Guanine aminohydrolase; Q9WTT6)	Guanine deaminase, hydrolase activity

effect reached significance only in the DPN-treated group (Fig. 3C). Unlike the negative regulation found in leukocytes (Fig. 3C), we discovered that expression of furin was not affected by any treatment in the uterus, where similar levels of mRNA were observed in all groups (Fig. 3D).

Calgranulin B was found to be up-regulated after DPN treatment (Table 4), but this effect was not verified by real-time PCR (Fig. 3E). The expression in uterus, though, was higher after PPT treatment than in the OvxC- and DPN-treated groups (Fig. 3F).

The fibulin-1 precursor was found to be down-regulated by E_2 treatment (Table 2). There was a tendency to down-regulation of the fibulin-1 mRNA found by PCR (Fig. 4A). Alternative splicing of fibulin-1 may result in four different variants. We investigated the expression of two predominant splice variants of fibulin-1 that can be discriminated by PCR, namely fibulin-1C and fibulin-1D. The expression of fibulin-1 variants C and D in leukocytes (Fig. 4C and E respectively) was similar to the fibulin-1 expression profile (Fig. 4A), but reached significant down-regulation for fibulin-1C after E_2 treatment and fibulin-1D after DPN treatment (Fig. 4C and E respectively). Fibulin-1 expression was significantly down-regulated by PPT treatment in the uterus (Fig. 4B), which is different to the leukocytes where the tendency to decrease was most pronounced in the E_2-treated group (Fig. 4A). Interestingly, we observed a different effect of treatments on the fibulin splicing variants in the uterus: fibulin-1C was induced by DPN, while fibulin-1D was suppressed by PPT (Fig. 4D and F respectively).

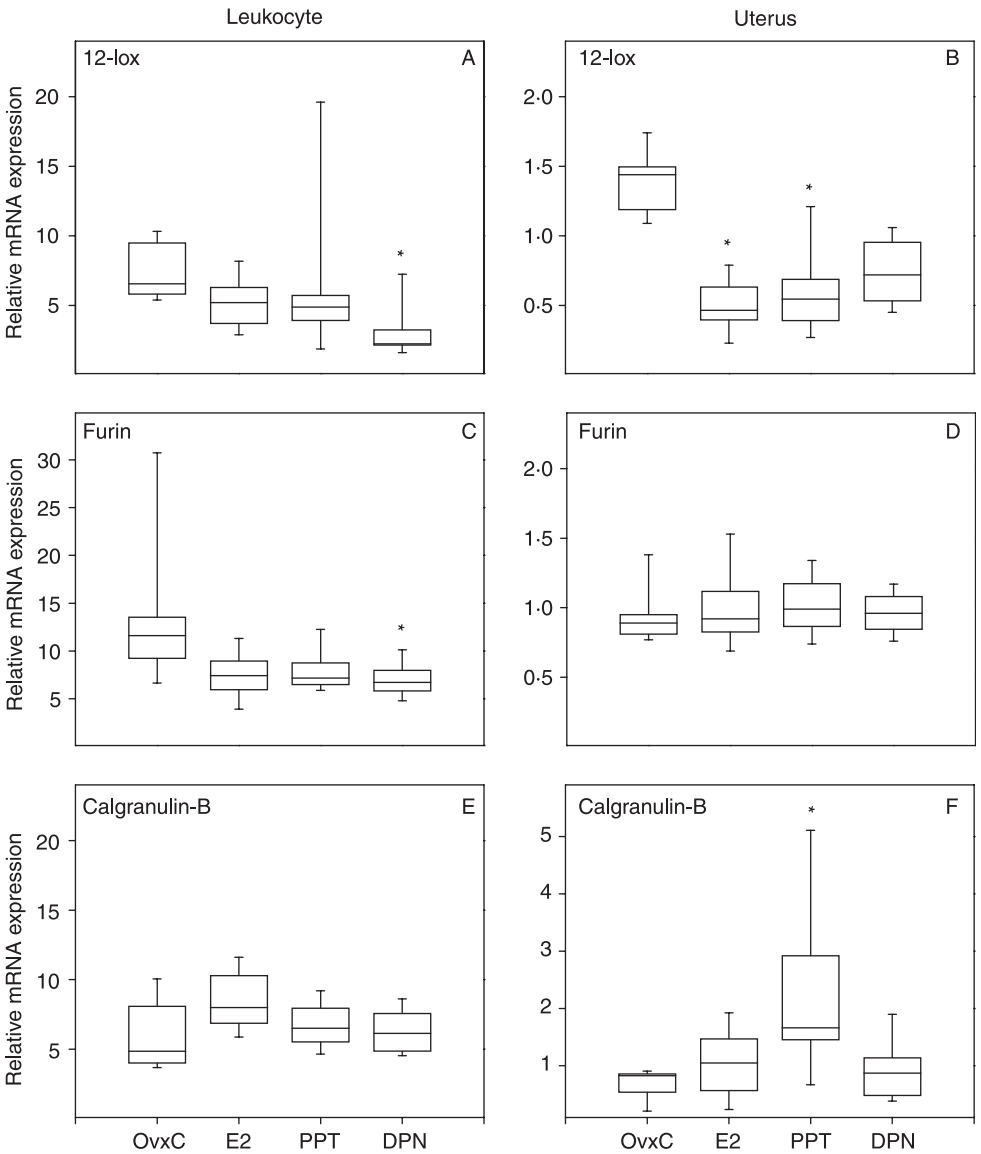

Figure 3 Representative real-time PCR experiments for 12-lox (A and B), furin (C and D), and calgranulin B (E and F) mRNAs expression in peripheral blood leukocytes (left column) and uterus (right column) from rats subjected to treatment with E_2, PPT, and DPN. The values of relative expression of target genes were normalized against HPRT and displayed in arbitrary units. Box and whisker plots represent the median value with 50% of all data falling within the box. The whiskers extend to the 5th and 95th percentiles. Group medians with asterisk superscripts are significantly different from control (OvxC).

Detection of 12-lox and calgranulin B in blood and uterus by western blot

The protein products of 12-lox and calgranulin B were studied by western blot analysis. The aim was to determine whether the selected regulated genes were also expressed on the protein level in leukocytes and uterus.

The western blot analysis confirmed that the 12-lox protein was present in peripheral blood leukocytes (Fig. 5A). A 60 kDa 12-lox protein was also detected in the uterus (Fig. 5A). The 12-lox protein bands were significantly stronger in the uteri of the PPT group when compared with controls (Fig. 5B).

Calgranulin B was identified in leukocytes as a 14 kDa band, and was also found in the uterus (Fig. 6). The bands were stronger in the uterus after E_2 and PPT treatment, whereas they were weak in the control and DPN groups (Fig. 6).

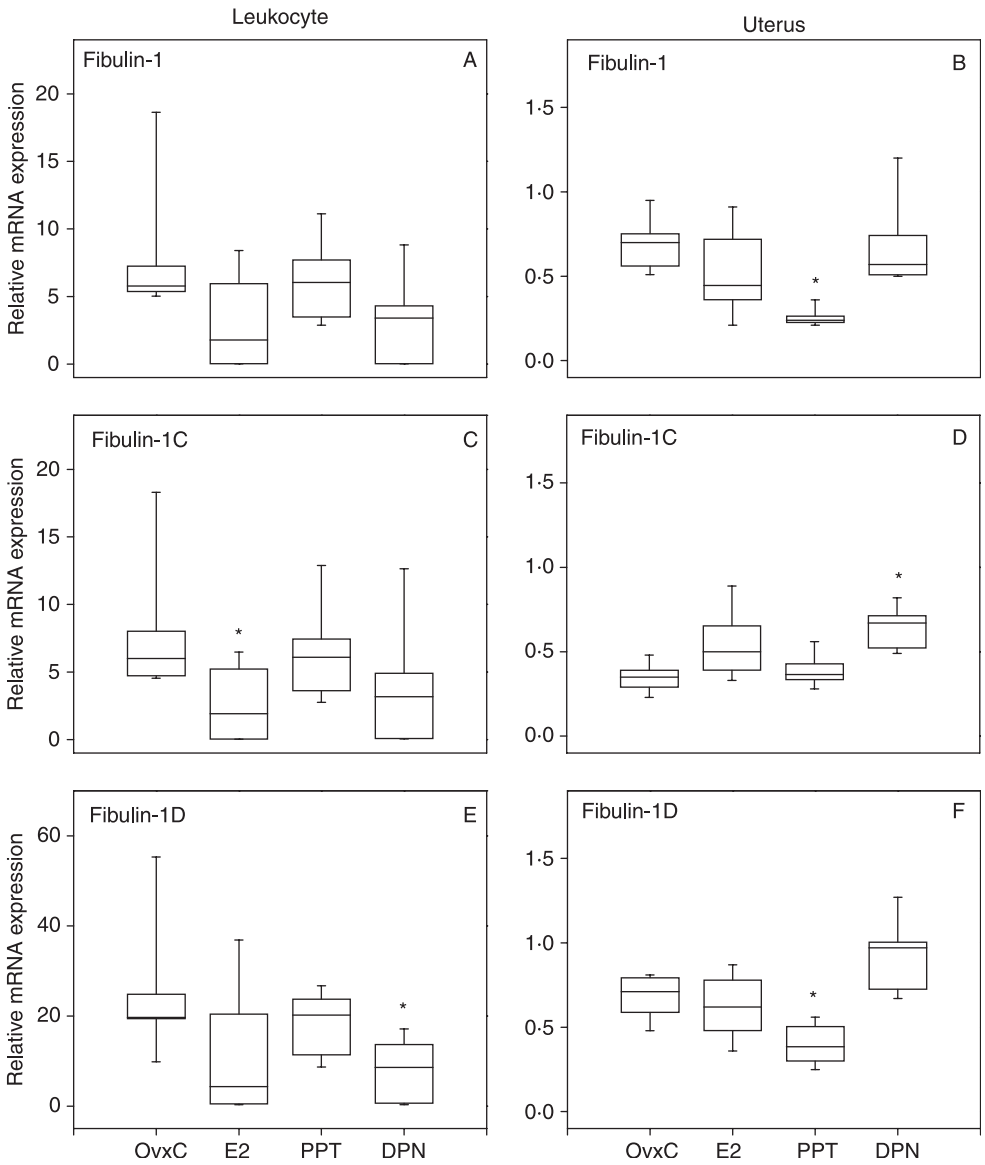

Figure 4 Representative real-time PCR experiments for fibulin-1 (A and B), fibulin-1C (C and D), and fibulin-1D (E and F) mRNAs expression in peripheral blood leukocytes (left column) and uterus (right column) from rats subjected to treatment with E_2, PPT, and DPN. The values of relative expression of target genes were normalized against HPRT and displayed in arbitrary units. Box and whisker plots represent the median value with 50% of all data falling within the box. The whiskers extend to the 5th and 95th percentiles. Group medians with asterisk superscripts are significantly different from control (OvxC).

Discussion

In this study, we investigated the activity of ERα and ERβ in peripheral blood leukocytes from ovx rats subjected to treatment with E_2, PPT, or DPN. Together with E_2, a biological agonist on both ERα and ERβ, we used the commercially available ER subtype-selective compounds PPT and DPN to highlight possible distinct roles for each ER isoform in leukocytes.

The recently developed non-steroidal compounds PPT and DPN were characterized as selective agonists for ERα and ERβ respectively. PPT is ~1000-fold potent as an agonist on ERα than on ERβ and has a 400-fold preference toward ERα in its binding affinity (Kraichely *et al.* 2000, Stauffer *et al.* 2000). DPN acts as an agonist on both ER subtypes, but has a 70-fold higher relative binding affinity and a 170-fold higher relative potency in transcription assays with ERβ than with ERα (Meyers *et al.* 2001).

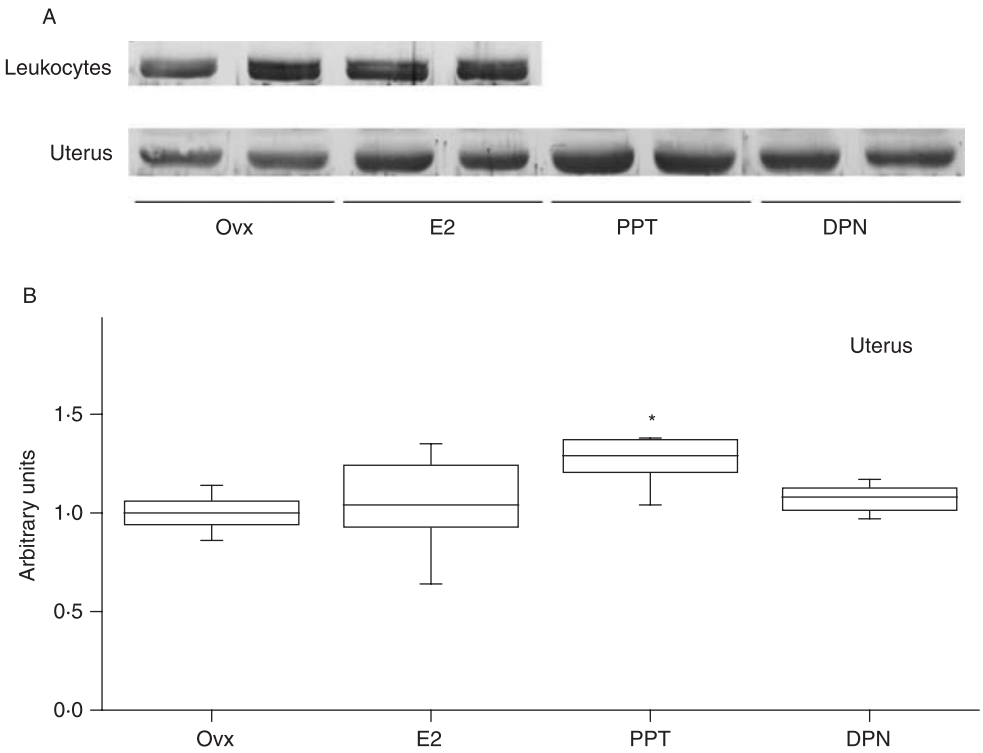

Figure 5 Representative western blot experiments for 12-lox (A) in rat peripheral blood leukocytes and uterus. Proteins were separated by SDS–PAGE and transferred to PVDF membranes, which were incubated with the primary antibody and AP-conjugated secondary antibody. The signal was detected using an AP substrate. The band corresponding to 12-lox is ~60 kDa in size. The bands from all animals were captured by a gel documentation system and used for statistical evaluation of the results (B). The PPT-treated group display stronger protein bands than the control group (B).

Microarray analysis showed that E_2 treatment resulted in transcriptional activation or repression of a large number of genes. Some of the genes, such as *annexin-1* and *bcl-x*, are known as estrogen-regulated genes (Castro-Caldas *et al*. 2001, Stoltzner *et al*. 2001). A few genes, including *mitochondrial uncoupling protein 2* and *platelet factor 4*, which were regulated by E_2 in our study, have been previously described as estrogen insensitive in other tissues (Norris & Bonnar 1994, Luukkaa *et al*. 2001, Stirone *et al*. 2005). We also detected transcriptional changes in a number of genes, which have not been reported to be estrogen-regulated (*leukocyte 12-lox*, *STAT6*, and *α-parvin*). The selective ERα agonist PPT and selective ERβ agonist DPN produced different effects on gene transcription. The majority of regulated genes were different in the E_2, PPT, and DPN treatment groups, pointing out different roles for ERα and ERβ. However, the transcriptional response in the selective agonists' groups was remarkably different when compared with the transcriptional changes in response to E_2 (as shown in Fig. 2), which could indicate that mechanisms of gene regulation by PPT and DPN may be substantially different from the mechanism of E_2 action. There is a possibility that the treatments themselves

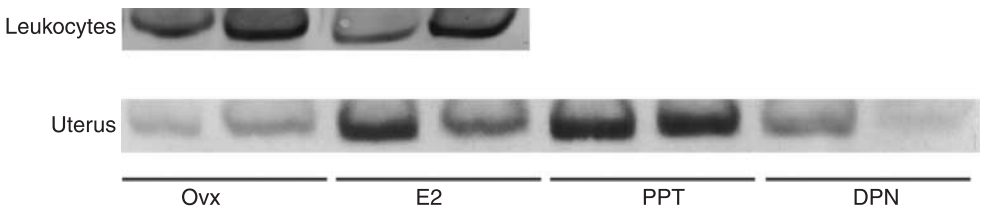

Figure 6 Representative western blot experiments for calgranulin B in rat peripheral blood leukocytes and uterus. Proteins were separated by SDS–PAGE and transferred to PVDF membranes, which were incubated with the respective primary antibodies and AP-conjugated secondary antibody. The signal was detected using an AP substrate. Calgranulin B was detected as a 14 kDa band. In the uterus, the calgranulin B bands from the E_2 and PPT treated animals are stronger than those of the control and DPN groups.

may have had an effect on the leukocyte pool, but due to the short treatment time, this effect would be limited.

Although the genes down-regulated by the three treatments are very different, as can be seen from the Venn diagrams, the functional annotation of the genes is more similar. They all down-regulate genes annotated primarily to categories 'cellular metabolism' and 'primary metabolism'.

From the enrichment analyses, we find that more than 50% of genes down-regulated by E_2 are related to 'binding' or 'cellular physiological processes', most significant are the enrichment of genes related to 'oxygen and reactive oxygen species metabolism' as well as 'response to oxidative stress'. In previous studies, we have shown that estrogens regulate expression of thioredoxin in rats (Sahlin *et al.* 1997*a*) and that thioredoxin and glutaredoxin mRNAs varies in human cervix and endometrium in relation to pregnancy and menstrual phase (Sahlin *et al.* 1997*b*, Stavréus-Evers *et al.* 2002). In pregnancies where prostaglandin E_2 was used to induce cervical ripening, glutaredoxin mRNA level was particularly increased (Sahlin *et al.* 1997*b*). In later studies, we have found that prostaglandin treatment induces leukocyte influx in the cervix (L Sahhin, Y Stjernholm-Vladic and G Ekman-Ordeberg, unpublished observations) and why the leukocytes might be the source of the glutaredoxin. Thus, the ERs in the leukocytes could mediate the increased glutaredoxin expression.

The enrichment analyses also showed that by DPN treatment the down-regulated genes were 56% related to 'intracellular' and that the most significantly changed genes were related to the Golgi network. Genes that were most significantly up-regulated by DPN related primarily to terms including 'ribosome' which could indicate that ERβ binding increases transcription. The up-regulated genes after DPN treatment were close to 30% related to 'immune response'. This finding could have impact on the sex difference and estrogen dependence found in morbidity and prevalence of autoimmune diseases (Druckmann 2001, Whitacre 2001).

Our study shows less overlap between genes regulated via ERα or ERβ than has been previously reported. Using the osteoblast-like cell line (U2OS) stably transfected with either ERα or ERβ, Stossi *et al.* (2004) demonstrated that 61% of all *E_2*-regulated genes were commonly regulated via *ERα* and *ERβ*. The difference in the proportion of commonly regulated genes does not seem to be dependent on the type of cells, as other groups studying the effect of E_2 on U2OS cell line reported only 17–21% of genes being commonly regulated via ERα and ERβ (Monroe *et al.* 2003, Kian Tee *et al.* 2004). The discrepancy may rather depend on experimental design and other variables such as duration of treatment.

In contrast to those studies, we investigated genes regulated by ERα and ERβ *in vivo* in different cell types expressing either one or both subtypes of ER. Interpretation of the results may be complicated because peripheral blood leukocytes contain several types of cells. Nevertheless, we deliberately avoided isolation of one or several cell types in order to be able to perform the analysis under true *in vivo* conditions. Thus, in the current study, no additional *ex vivo* procedures, such as incubations and density gradient centrifugations, were performed. These procedures have been shown to change gene expression pattern dramatically (Riches *et al.* 1992, Hartel *et al.* 2001). Instead, we stabilized RNA immediately on blood collection, allowing accurate *in vivo* investigation of gene expression profile in leukocytes, as most of the mRNA in whole blood is considered to originate from leukocytes. A disadvantage with using whole blood RNA is that it may contain relatively high amounts of globin mRNA, which may reduce the sensitivity of microarray analysis.

For validation of microarray results, we chose four genes that are considered to be involved in ECM remodeling and inflammatory response. These genes were *arachidonate 12-lox, fibulin-1, furin,* and *calgranulin B. Leukocyte type 12-lox* of the rat is similar to *human 15-lox*. *Arachidonate 12-lox* has enzymatic activity and is involved in one of the two major metabolic pathways (lipoxygenase and cyclooxygenase) of arachidonic acid. As the 12-lox products are present on the site of inflammation, this enzyme was previously believed to exhibit pro-inflammatory activities (reviewed in Yoshimoto & Takahashi 2002). In recent years, several lines of experimental evidence suggest that 12-lox products might also exhibit anti-inflammatory properties (Levy *et al.* 2001, Vachier *et al.* 2002, Serhan *et al.* 2003). Increased concentrations of arachidonic acid 12-lox metabolites (12-hydroxyeicosatetraenoic acid, leukotriene B4) are observed in amniotic fluid during parturition suggesting a role in active labor (Romero *et al.* 1987). Moreover, 12-lox metabolites may be involved in the ECM remodeling as suggested by increased production of fibronectin and collagens in certain cell lines overexpressing 12-lox (Wen *et al.* 2003).

In our study, the effect of the treatments on *12-lox* expression was different in the leukocytes when compared with the uterus. The negative regulation of uterine 12-lox mRNA was most prominent in the E_2 and PPT groups, while in the leukocytes, the greatest decrease was found in the DPN-treated group. Assuming that the *12-lox* expression is regulated via ERα and ERβ in a similar manner, the variation between responses to the selective agonists in different tissues may depend on differences in the ratio of ERα/ERβ. It is recognized that the uterine tissue expresses predominantly ERα, while ERβ is thought to be the dominating subtype in leukocytes (Wang *et al.* 1999, Stygar *et al.* 2001). It is striking though that the 12-lox protein is up-regulated by ERα activity in the uterus as determined by western blot. The rationale for this is not known, but post-transcriptional regulation seems to be more powerful than the transcriptional regulation of the gene expression itself.

Another gene selected for validation was *fibulin-1*. Fibulins are a family of proteins associated with basement membranes and elastic ECM fibers (reviewed in Argraves *et al.* 2003). They are hypothesized to function as intra-molecular bridges that stabilize the organization of supra-molecular ECM

structures. Fibulin-1 is a cofactor of the ADAMTS-1 metalloprotease which is active in proteolysis of the proteoglycan versican, thus involved in ECM remodeling (Kern *et al.* 2006). Fibulin-1 shows fibronectin- and integrin-binding properties and the level of this protein in the endometrium fluctuates during the menstrual cycle. It has been demonstrated that fibulin-1 is regulated by sex steroid hormones. Haendler *et al.* (2004) reported that the fibulin-1 gene in human endometrium is regulated both by estrogen and progesterone and that the effect appears to be cell-type specific. E_2 induced fibulin-1 in endometrial glands, while the inductive effect of progesterone was observed in the endometrial stroma. In ER-positive ovarian and breast cancer cell lines, fibulin-1 mRNA levels are markedly increased by estrogens. Transfection experiments using fibulin-1 promoter constructs demonstrated that E_2 increases fibulin-1 gene transcription and that ERα is more potent than ERβ to mediate E_2 regulation of the transfected fibulin-1 promoter (Bardin *et al.* 2005). In our study, *fibulin-1* was identified among genes down-regulated by E_2 in leukocytes and this finding was consistent when validated with real-time PCR. In the uterus, we report differential regulation of *fibulin-1* splice variants C and D by the selective ERα and ERβ agonists. Induction of *fibulin-1C* by DPN is contrasted by suppression of *fibulin-1D* by PPT, suggesting that the transcriptional effect of ERβ opposes the effect of ERα. It is obvious that the *fibulin-1* gene is under hormonal control, but the exact mechanism of regulation and function of this protein in the ECM need further studies.

Furin was identified among regulated genes by the microarray and selected for further analysis. Furin is one of the proteolytic enzymes activating MMP zymogens (Verma & Hansch 2007), and thus involved in ECM remodeling. Furin is a calcium-dependent serine protease of the subtilisin-like pro-protein convertase family. This transmembrane glycoprotein catalyses the maturation of a very diverse group of bioactive proteins, ranging from growth factors and receptors to pathogen proteins (reviewed in Molloy *et al.* 1999). Furin is also efficient at cleaving several adhesion-related proteins involved in cell–ECM interactions. In addition, furin processes protein substrates such as pro-membrane-type-1 MMPs (proMT1-MMP) and pro-transforming-growth-factor β, which play important roles in the remodeling of ECM and basement membranes (Dubois *et al.* 1995, Sato *et al.* 1996). We demonstrate that gene expression of *furin* in leukocytes is negatively regulated by estrogenic substances, with the most profound effect found in the DPN treatment group. No effect was found on the *furin* expression in the uterus. Once again, our differing results between leukocytes and uterus could depend on the dominant ER subtype present, i.e. ERβ in leukocytes and ERα in uterus (Wang *et al.* 1999, Stygar *et al.* 2001).

Calgranulin B (MRP14 or S100A9) is a potent stimulator of neutrophils and the protein is suggested to be involved in neutrophil migration to inflammatory sites (Ryckman *et al.* 2003) and it is also a substrate for MMP-2 and MMP-9 (Greenlee *et al.* 2006), and thus likely to be involved in ECM remodeling. Calgranulin B is the major calcium-binding protein of neutrophils and monocytes (Vogl *et al.* 2004), and stimulates proliferation of fibroblasts, suggesting that it plays a role in chronic inflammation (Shibata *et al.* 2004). The cellular properties of calgranulin B, its spatial localization, and dramatic increase in cervix and myometrium of women in labor suggest that this protein may be very important in the initiation or propagation of human labor (Havelock *et al.* 2005). In contrast to that, studies of MRP-14(−/−) mice showed that they have no obvious phenotype and are fertile (Hobbs *et al.* 2003). Our data on calgranulin B regulation in leukocytes by DPN treatment as found by the microarray were not confirmed by real-time PCR, although there was a tendency toward increasing levels after estrogen treatment. In the uterus, however, the level was up-regulated by PPT treatment. By western blot, we found varying calgranulin B protein levels in the leukocytes, both in controls and after E_2 treatment. Though, in the uterus, E_2 and PPT treatment seemed to increase the protein level, indicating regulation via predominantly ERα, well in agreement with the PCR results.

The selected genes were often differently regulated comparing leukocytes and uterus from the same animals. The transcription is regulated by enhancers and repressors (co-regulators), which may differ depending on the cell type and tissue (O'Malley 2007). Thus, gene regulation by receptor activation is regulated by far more factors than the actual ligand, and these co-regulators will modify the effect due to tissue and cell type.

Our data describe gene expression profiles measured at a single time point and after a single dose of treatment. Thus, it is possible that additional forms of gene regulation may arise if other doses and time points are used.

Two of the selected genes were also analyzed on a protein level and were both detected in the leukocytes by western blot. The changes in the protein levels of 12-lox in uterus did not follow the gene regulation pattern. A possible explanation to the discrepancy between mRNA and protein levels is that many other genes are regulated by ER activation and some of these gene products could affect protein stability and turnover rate.

In recent years, studies have shown evidence for both pro- and anti-inflammatory activities of estrogens. The key cells in the inflammatory reaction are leukocytes. Our study demonstrates that peripheral blood leukocytes are responsive to estrogens. E_2 and the selective ERα and ERβ agonists regulate a number of genes that may contribute to remodeling of ECM and inflammation. The effect on mRNA levels is predominantly negative suggesting that pro-inflammatory and catabolic activities of leukocytes are likely to be reduced by estrogens. On the other hand, the impression from protein levels of the same gene products implies that the protein does not

always reflect the effect on the mRNA level. Thus, the final effect from estrogen treatment is multi-regulated; directly on the gene expression level, on the post-transcriptional level and possibly also indirectly, via other proteins. This could be a major reason for the various effects from estrogen treatment that has been presented, the multitude in regulatory effects depend on dose, time, administration method, and cell or tissue type studied.

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