

Endothelium and Vascular Development

The estrogen metabolite 17 β -dihydroequilenin counteracts interleukin-1 α induced expression of inflammatory mediators in human endothelial cells *in vitro* via NF- κ B pathway

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Summary

In most studies showing cardio- and vasculoprotective effects of estrogens, 17 β -estradiol was used and little information on possible effects of different estrogen metabolites is yet available. We investigated whether particular estrogen metabolites are effective in counteracting inflammatory activation of human endothelium. Human endothelial cells were incubated with 17 α -dihydroequilenin, 17 β -dihydroequilenin, δ -8,9-dehydroestrone, estrone and 17 β -estradiol and stimulated with interleukin (IL)-1 α . The expression of IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) was determined. 17 β -dihydroequilenin and 17 β -estradiol at a concentration of 1 μ M reduced IL-1 α -induced up regulation of IL-6, IL-8 and MCP-1 close to control levels. When both compounds were used in com-

ination an additive effect was observed. 17 α -dihydroequilenin and δ -8,9-dehydroestrone showed a similar anti-inflammatory effect only when used at 10 μ M whereas estrone had no effect. The effect of 17 β -dihydroequilenin on IL-1 α -induced production of IL-6, IL-8 and MCP-1 was reversed by the estrogen receptor antagonist ICI 182,780. 17 β -dihydroequilenin also inhibited IL-1 α -induced translocation of p50 and p65 to the nucleus of the cells. We have identified the estrogen metabolite 17 β -dihydroequilenin, as an inhibitor of inflammatory activation of human endothelial cells. Characterization of specific estrogens – as shown in our study – could provide the basis for tailored therapies, which might be able to achieve vasoprotection without adverse side effects.

Keywords

Estrogens, endothelial cells, cardiovascular effects, inflammation, cytokines

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Introduction

Cardiovascular disease is the leading cause of death in the Western world (1). Epidemiological studies have shown that the incidence of cardiovascular disease in premenopausal women is lower than in men, but that it reaches a similar value after menopause (2). These findings have suggested a role for estrogens in modulating susceptibility to cardiovascular disease. This notion is supported by numerous studies that have shown cardioprotective effects of estrogens, which only partially can be attributed to their favourable modification of the lipid profile (3). In fact recent evidence suggests various direct vascular effects of estrogens (4). In animal studies 17 β -estradiol was shown to maintain endothelial integrity, to prevent leukocyte adhesion and to reduce neointima formation (5–7). *In vitro* studies have demonstrated that estradiol has anti-inflammatory potential by significantly inhibiting the expression of interleukin-6 (IL-6), IL-8, monocyte chemoattractant protein-1 (MCP-1) and adhesion

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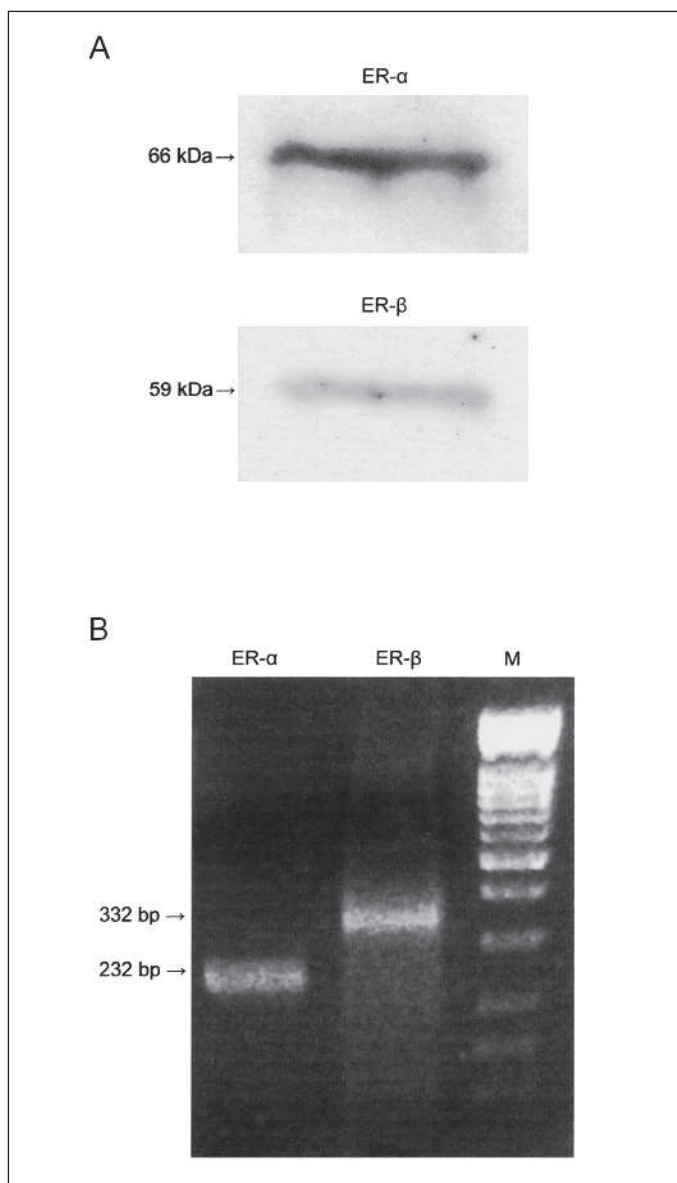


Figure 1: Expression of estrogen receptors α and β in HUVECs: Nuclear extracts of HUVECs were Western blotted and estrogen receptor- α (ER- α) and ER- β were visualized using specific antibodies as described in the Materials and Methods section (panel A). RT-PCR performed with specific primers as described in the Materials and Methods section showed the expression of ER- α and ER- β specific mRNA in HUVECs (panel B).

molecules in endothelial cells (8–12). Furthermore estrogens stimulate the production of nitric oxide (NO) in endothelial cells and protect these cells from injury caused by hypoxia/reoxygenation (13, 14). A recent publication has shown that estrogens increase bone marrow-derived endothelial progenitor cell production and thus accelerate vascular repair (15). This epidemiological and experimental evidence, however, is contrasted by findings of randomized hormone replacement therapy trials for the primary and secondary prevention of cardiovascular disease (16, 17). The lack of protective effects of estrogens in the latter studies might be attributed to the differences in chemical charac-

teristics of conjugated equine estrogens used and the co-administration of a synthetic progestin (18). Thus it seems of considerable interest to characterize different estrogen metabolites with regard to their beneficial or adverse effects on mechanisms that play a major role in cardiovascular disease. In most of the experimental studies mentioned above, however, 17 β -estradiol was used and little information on possible effects of different estrogen metabolites is yet available. Only recently Dubey et al. described that tibolone and its metabolite inhibit mitogen-induced growth of vascular smooth muscle cells and Mukherjee et al. presented evidence that 17-epiestriol, an estrogen metabolite, is more effective in inhibiting the expression of vascular cell adhesion molecule-1 (VCAM-1) in inflammatory activated human endothelial cells than 17 β -estradiol (19, 20).

To further address this question we studied whether the estrogen metabolites 17 α -dihydroequilenin, 17 β -dihydroequilenin, δ -8,9-dehydroestrone, estrone, and 17 β -estradiol, which are active compounds of Premarin commonly used to treat postmenopausal women (21), are effective in counteracting inflammatory activation of human endothelium. For this purpose endothelial cells were activated with IL-1 α and possible anti-inflammatory effects of different estrogen metabolites on the expression of the inflammatory mediators IL-6, IL-8 and MCP-1 in such activated cells were compared.

Materials and methods

Cell culture

HUVEC were isolated from fresh umbilical cords by mild collagenase treatment, cultured and characterized as described (22). In order to avoid possible estrogen-like effects of phenol red, cultures were switched to a phenol red-free minimum essential medium (M199; Sigma, St. Louis, USA) 72 hours (h) before each experiment. Before treatment with estrogens cells were pre-incubated over 24 h with 20% activated carbon absorbed fetal calf serum (FCS, PAN Biotech GmbH, Germany) not containing any endogenous estrogens. All cells used in this study were between passage 3 and 6. All human material was obtained and processed according to the recommendations of the hospital's Ethics Committee and Security Board.

Treatment of cells with estrogens

The following estrogen metabolites were evaluated 17 α -dihydroequilenin, 17 β -dihydroequilenin, δ -8,9-dehydroestrone, 17 β -estradiol and estrone. 17 β -estradiol was obtained from Sigma (St. Louis, USA). The other compounds were kindly provided by Wyeth-Ayerst Women's Health Research Institute (Philadelphia, PA). Initially HUVECs were incubated for 24 h in 1,25% activated carbon absorbed FCS without or with the estrogen metabolites or a combination of 17 β -dihydroequilenin and 17 β -estradiol at the indicated concentrations. Human recombinant IL-1 α (R&D Systems, Minneapolis, USA) was then added to the cells to give a final concentration of 200 U/ml and the cultures were incubated for a further 24 h. In additional experiments HUVECs were pre-treated for 60 minutes with or without 100 μ M of pure estrogen receptor antagonist ICI 182,780 (Tocris Cookson; Ballwin, USA) followed by 24 h incubation with 1 μ M 17 β -dihydroequilenin. To some of these cultures

Table 1: Primers used for RT-PCR and RealTime-PCR analysis.

Target	fwd-Primer (corresponding position)	rev-Primer (corresponding position)	Annealing temperature (Amplicon Size [bp])
GAPDH	5'-ACA GTC CAT GCCATCACT GCC-3' (604 – 625)	5'-GCC TGC TTC ACC ACC TTC TTG-3' (890 – 869)	65°C 286
IL-6	5'-CAG TAC CCC CAG GAG AAG AT-3' (148 – 167)	5'-GCA GGA ACT GGA TCA GGA C-3' (525 – 507)	65°C 378
IL-8	5'-ACC GGA AGG AAC CAT CTC AC-3' (44 – 63)	5'-CTT GGG GTC CAG ACA GAG C-3' (317 – 299)	68°C 274
MCP-1	5'-ACT GAA GCT CGCACT CTC-3' (45 – 62)	5'-CTT GGG TTG TGG AGT GAG-3' (392 – 375)	65°C 348
ER- α	5'-GAT GAA TCT GCA GGG AGA GG-3' (1671 – 1690)	5'-GAT GTG GGA GAG GAT GAG GA-3' (1902 – 1883)	60°C 232
ER- β	5'-TGT GCG GAG ACA GAG AAG TG-3' (1102 – 1121)	5'-ACA CCT CCA TCC AAC AGCT C-3' (1433 – 1414)	60°C 332

IL-1 α at a concentration of 200 U/ml was added after 18 h. The culture supernatants were then collected followed by removal of cell debris by centrifugation and stored at -80°C until used. The total cell number of the respective cultures after trypsinisation was counted with a haemocytometer.

ELISA

IL-6, IL-8 and MCP-1 proteins were measured in supernatants using specific enzyme-linked immunosorbent assays (ELISAs) using monoclonal antibodies (IL-6 eBioscience, San Diego, USA; IL-8 and MCP-1 Module Sets, Bender MedSystems Diag-

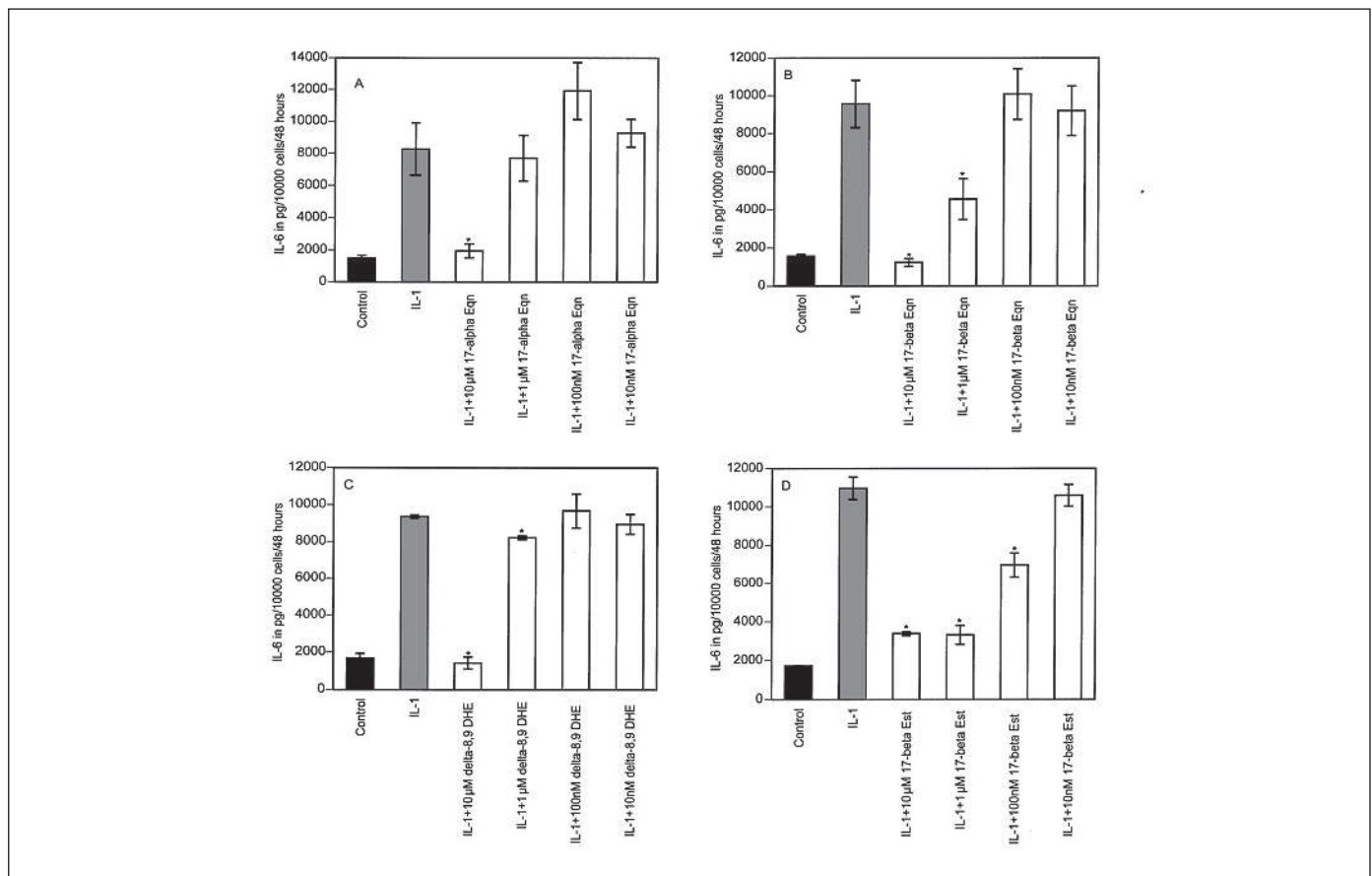


Figure 2: Effects of 17 α -dihydroequilenin (panel A), 17 β -dihydroequilenin (panel B), δ -8,9-dehydroestrone (panel C) and 17 β -estradiol (panel D) on IL-1 α -induced production of IL-6 in HUVECs: HUVECs were incubated for 24 hours without or with the respective metabolite at the indicated concentration. Thereafter human recombinant IL-1 α was added to the cells to give a final concentration of

200 U/ml and the cultures were incubated for a further 24 hours. Conditioned media of such treated cells were collected and IL-6 was determined as described in the Materials and Methods section. Values are given in pg/10⁴ cells/48 hours and represent mean values \pm SD of three independent determinations. Experiments were performed at least three times. A representative experiment is shown. *p<0.05

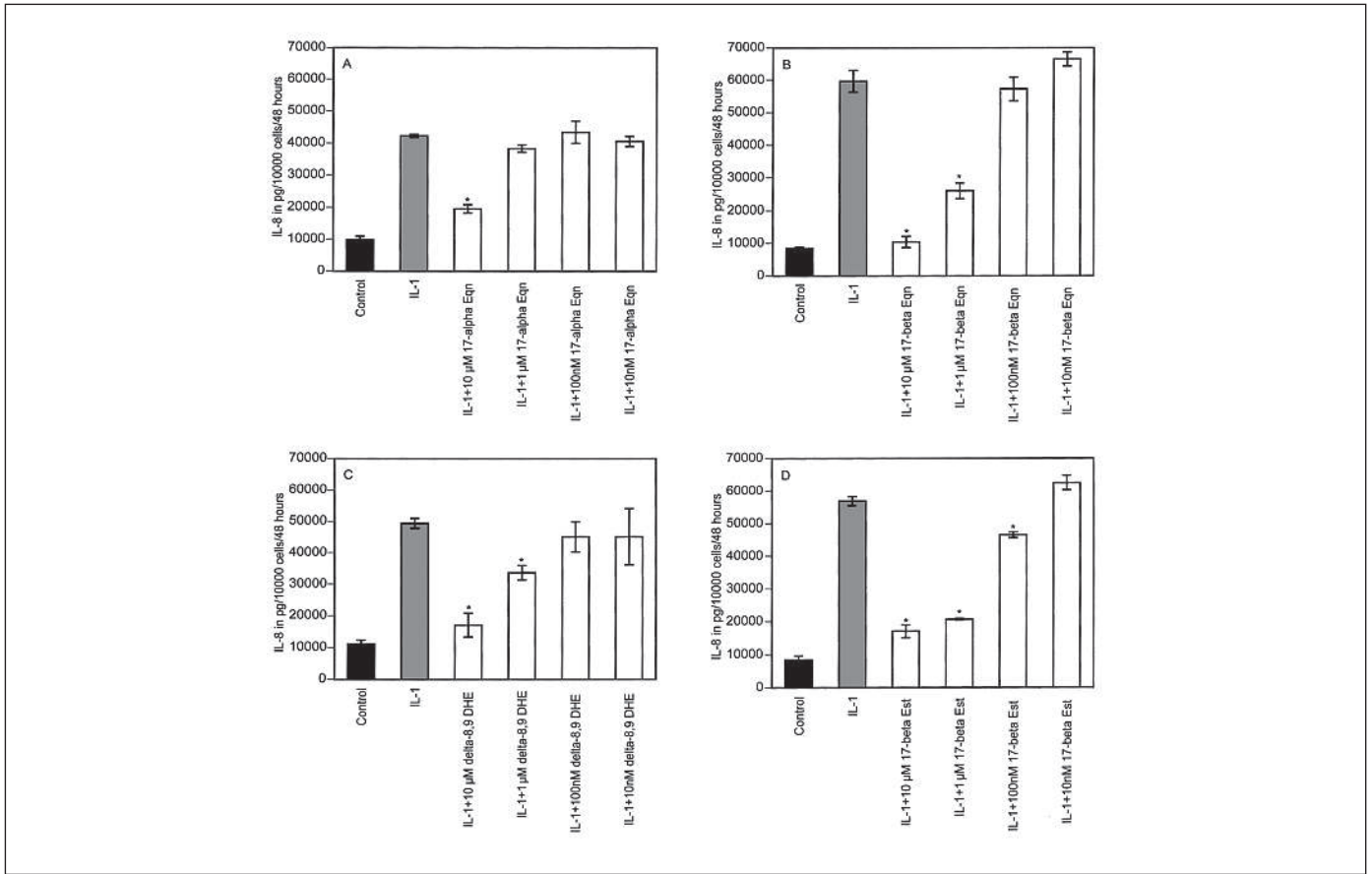


Figure 3: Effects of 17 α -dihydroequilenin (panel A), 17 β -dihydroequilenin (panel B), δ -8,9-dehydroestrone (panel C) and 17 β -estradiol (panel D) on IL-1 α -induced production of IL-8 in HUVECs: HUVECs were incubated for 24 hours without or with the respective metabolite at the indicated concentration. Thereafter human recombinant IL-1 α was added to the cells to give a final concentration of

200 U/ml and the cultures were incubated for a further 24 hours. Conditioned media of such treated cells were collected and IL-8 was determined as described in the Materials and Methods section. Values are given in pg/10⁴ cells/48 hours and represent mean values \pm SD of three independent determinations. Experiments were performed at least three times. A representative experiment is shown. *p < 0.05

nostics GmbH, Vienna, Austria). All measurements were performed in triplicates.

mRNA purification

Cells were stimulated as described, supernatants were removed and mRNA was isolated using QuickPrepTM Micro mRNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions.

RealTime-Polymerase Chain Reaction

Specific mRNA levels for IL-6, IL-8 and MCP-1 were determined by RealTime-Polymerase Chain Reaction (RealTime-PCR) using LightCycler-RNA Master SYBR Green I (Roche, Basel, Switzerland) according to the manufacturers instructions. Primers, shown in Table 1, were designed using the LightCycler Probe Design Software Version 1.0 and the Primer3 Software. The amplification conditions consisted of an initial incubation at 61°C for 20 minutes, followed by incubation at 95°C for 30 seconds, 50 cycles of 95°C for 1 second, the annealing temperature (65°C for IL-6 and MCP-1, and 68°C for IL-8) for 10 seconds and 72°C for 10 seconds, a melting step from 45°C to 95°C increasing 0.1°C per second and a final cooling to 40°C. Data was

analysed using LightCycler Software Version 3.5 (Roche, Basel, Switzerland).

Reverse Transcriptase Polymerase Chain Reaction

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed using Titan One Step RT-PCR System (Roche, Basel, Switzerland) according to the manufacturers instructions. The amplification conditions consisted of an initial incubation at 50°C for 30 minutes, followed by incubation at 94°C for 2 minutes, 30 cycles of 94°C for 30 seconds, the annealing temperature of 60°C for both estrogen receptor- α (ER- α) and ER- β for 45 seconds and 68°C for 1 minute, and a final incubation at 68°C for 7 minutes. PCR-Products were analysed by gel-electrophoresis (3% Agarose-gel, stained with ethidiumbromide). Primers, shown in Table 1, were designed using the Primer3 Software (http://www-genome.wi.mit.edu/genome_software/other/primer3.html).

Nuclear extraction and analysis of NF- κ B/DNA binding

Initially HUVECs were incubated for 24 h in 1,25% activated carbon absorbed FCS with or without 17 β -dihydroequilenin at concentrations of 100nM and 1 μ M. Human recombinant IL-1 α was then added to the cells to give a final concentration of 200

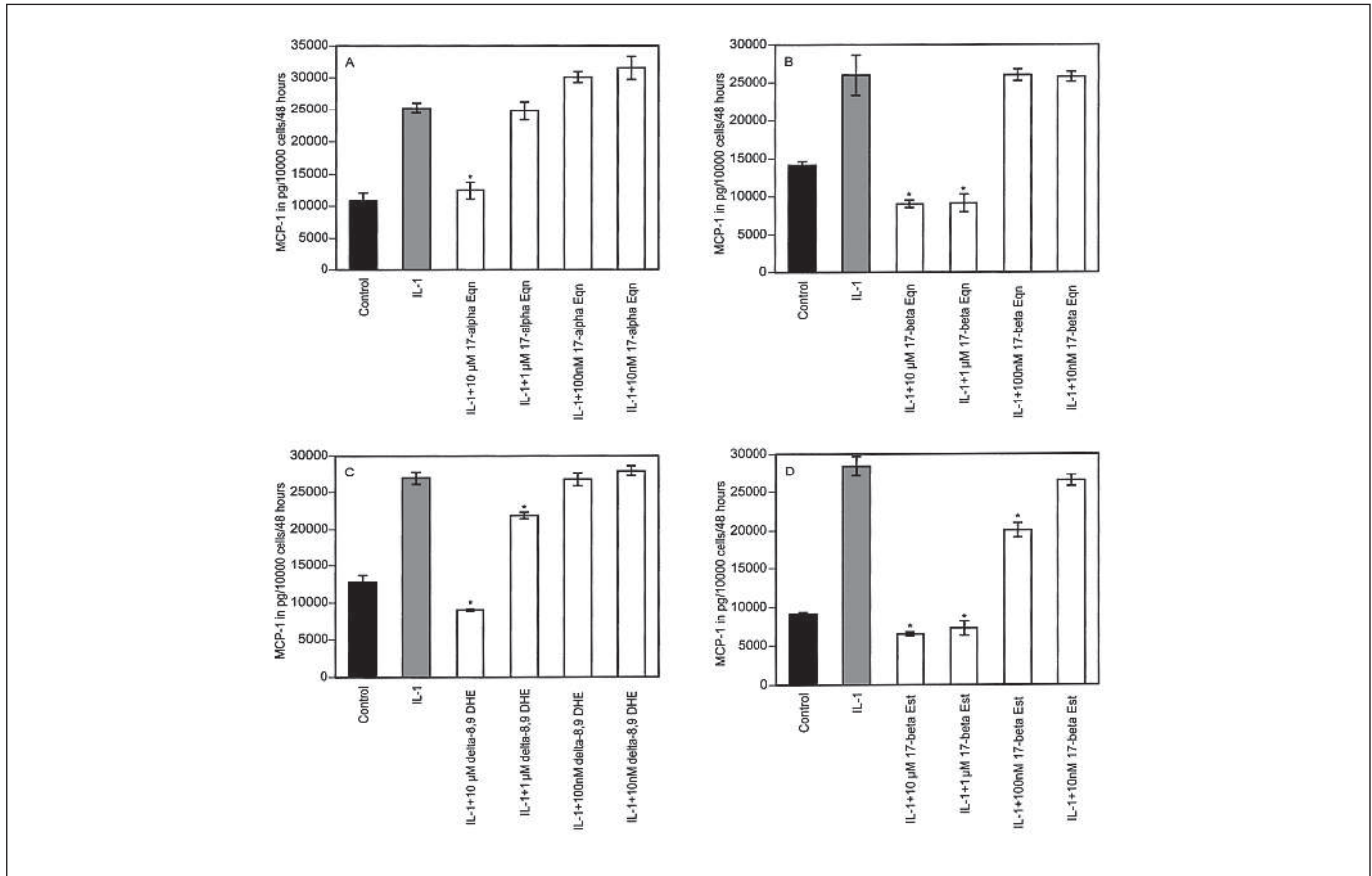


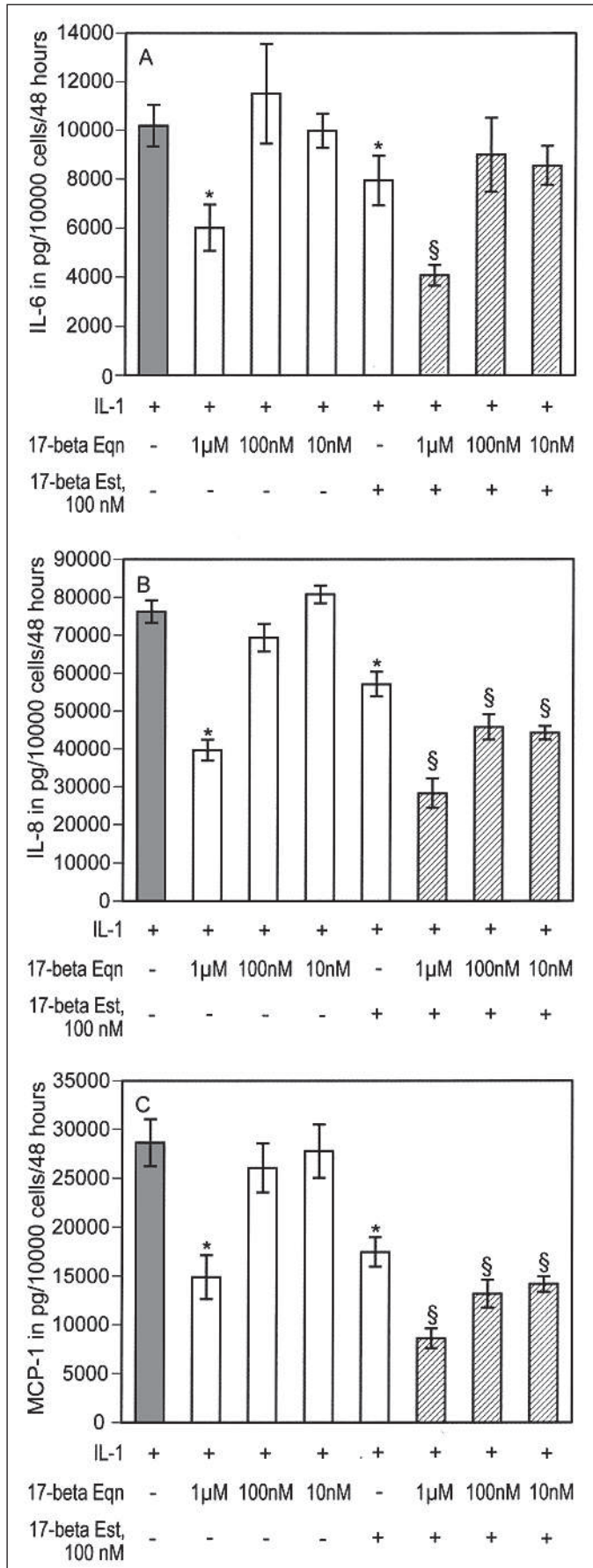
Figure 4: Effects of 17 α -dihydroequilenin (panel A), 17 β -dihydroequilenin (panel B), δ -8,9-dehydroestrone (panel C) and 17 β -estradiol (panel D) on IL-1 α -induced production of MCP-1 in HUVECs: HUVECs were incubated for 24 hours without or with the respective metabolite at the indicated concentration. Thereafter human recombinant IL-1 α was added to the cells to give a final concentration of 200 U/ml and the cultures were incubated for a further 24 hours. Conditioned media of such treated cells were collected and MCP-1 was determined as described in the Materials and Methods section. Values are given in pg/10⁴ cells/48 hours and represent mean values \pm SD of three independent determinations. Experiments were performed at least three times. A representative experiment is shown. * $p < 0.05$

and the cultures were incubated for a further 2 hours. Preparation of nuclear extracts was performed by Nuclear Extract Kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions. Quantitation of NF- κ B in nuclear extracts of such treated HUVECs was performed using the ELISA-based TransAMTM NF- κ B Family kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instructions. Briefly, the active NF- κ B contained in nuclear extracts specifically bound to an oligonucleotide immobilized to a 96-well plate, which contains an NF- κ B consensus binding site. By using specific antibodies directed against the NF- κ B p50 and p65 subunits, the NF- κ B subunits bound to the oligonucleotide were detected. The amount of bound p50 and p65 was quantified by spectrophotometry using a secondary antibody conjugated to horseradish peroxidase. Specificity of p50 and p65 binding was confirmed by incubation of nuclear extracts with the immobilized NF- κ B consensus probe in the presence of excess wild type or mutated oligonucleotide.

Western Blotting
Nuclear and cytoplasmic cell fractions were prepared from freshly frozen HUVECs using a commercially available nuclear

and cytoplasmic extraction reagent kit (NE-PER[®], Pierce, Rockford, IL) according to the manufacturer's instructions. The extracts were then stored at -80°C until further use. Exact protein concentrations were determined by spectrophotometry using the MicroBCA-Protein Assay Reagent kit from Pierce. 25 μg of nuclear protein extracts were separated by SDS-polyacrylamide-gel electrophoresis on ExelGel 8–18% gradient gels (AP Biotech, Uppsala, Sweden) and blotted on a Nitrocellulose-membrane (AP Biotech). Membranes were blocked in PBS containing 2.5% nonfat dry milk, 2.5% BSA (Sigma), and 0.05% Tween 20 (Promega, Madison, USA) for at least one hour. Immunoreactions were performed either with an anti-ER α (AB-10, Neomarkers, Fremont, USA) or anti-ER β (6B12, Genetex, San Antonio, USA) monoclonal antibody (final concentrations of both ABs: 1 $\mu\text{g}/\text{ml}$). Specificity of antibodies used for Western blot has been shown previously by absorption experiments on recombinant human ER α and ER β protein (23).

Western blot analysis of NF- κ B subunit p65 nuclear translocation was performed on nuclear extracts as described by us previously using an antibody against the NF- κ B subunit p65 obtained from Santa Cruz Biotechnology (Santa Cruz, USA) (24).



Determination of cell viability

In order to determine possible cytotoxic effects of estrogens, lactate dehydrogenase (LDH) leakage was measured in cultures treated with estrogens metabolites as described above using a commercially available assay for photometric determination of LDH activity (Sigma, St. Louis, USA). Cell viability was not significantly altered by estrogens in HUVECs at concentrations used in the experiments described.

Statistical analysis

Values are expressed as mean \pm SD. Data were compared by ANOVA. Values of $p < 0.05$ were considered significant.

Results

Expression of estrogen receptors α and β in HUVECs

HUVECs expressed ER- α and ER- β both on a protein level as well as on the level of specific mRNA as determined by Western blotting and RT-PCR (Fig. 1).

Effects of estrogens on IL-1 α -induced IL-6, IL-8 and MCP-1 protein production in HUVECs

IL-1 α increased the production of IL-6 in HUVECs up to 6-fold. 17 β -dihydroequilenin and 17 β -estradiol used at a concentration of 1 μ M down regulated IL-1 α -stimulated IL-6 production almost to control levels, whereas 17 α -dihydroequilenin, and δ -8,9-dehydroestrone showed this capacity only at a concentration of 10 μ M. Also δ -8,9-dehydroestrone slightly, but significantly down regulated IL-6 production when used at a concentration of 1 μ M. 17 β -estradiol was effective also at a concentration of 100nM (Fig. 2). In contrast estrone had no significant effect on IL-1 α -induced up regulation of IL-6 at any concentration used (data not shown).

Similar to results described above, IL-1 α also increased the production of IL-8 in HUVECs up to 7-fold. Again 17 β -dihydroequilenin and 17 β -estradiol used at a concentration of 1 μ M down modulated IL-1 α -induced IL-8 production close to control levels, whereas 17 α -dihydroequilenin, and δ -8,9-dehydroestrone showed this capacity only at a concentration of 10 μ M. Also δ -8,9-dehydroestrone slightly, but significantly down regulated IL-8 production when used at a concentration of 1 μ M. 17 β -estradiol was effective also at a concentration of 100nM (Fig. 3). Es-

Figure 5: Effects of 17 β -dihydroequilenin in combination with 17 β -estradiol on IL-1 α -induced production of IL-6 (panel A), IL-8 (panel B) and MCP-1 (panel C) in HUVECs: HUVECs were incubated for 24 hours without or with the two compounds alone or in combination at the indicated concentrations. Thereafter human recombinant IL-1 α was added to the cells to give a final concentration of 200 U/ml and the cultures were incubated for a further 24 hours. Conditioned media of such treated cells were collected and IL-6, IL-8 and MCP-1 was determined as described in the Materials and Methods section. Values are given in pg/10⁴ cells/48 hours and represent mean values \pm SD of three independent determinations. Experiments were performed at least three times. A representative experiment is shown. * $p < 0.05$ as compared to HUVECs treated with IL-1 α alone; $\$p < 0.05$ as compared to HUVECs treated with IL-1 α in combination with 17 β -dihydroequilenin or 17 β -estradiol alone at the corresponding concentration

trone had no significant effect on IL-1 α -induced up regulation of IL-8 at any concentration used (data not shown).

As it was the case for IL-6 and IL-8, IL-1 α also increased the production of MCP-1 in HUVECs significantly. The magnitude of the increase, however, was lower (up to 3-fold). 17 β -dihydroequilenin and 17 β -estradiol used at a concentration of 1 μ M again down regulated IL-1 α -induced MCP-1 production to control levels, whereas 17 α -dihydroequilenin, and δ -8,9-dehydroestrone showed this effect only at a concentration of 10 μ M. Similar to the results described above for IL-6 and IL-8, δ -8,9-dehydroestrone slightly, but significantly also down regulated MCP-1 production when used at a concentration of 1 μ M. 17 β -estradiol was effective also at a concentration of 100nM (Fig. 4). Estrone had no significant effect on IL-1 α -induced up regulation of MCP-1 at any concentration used (data not shown).

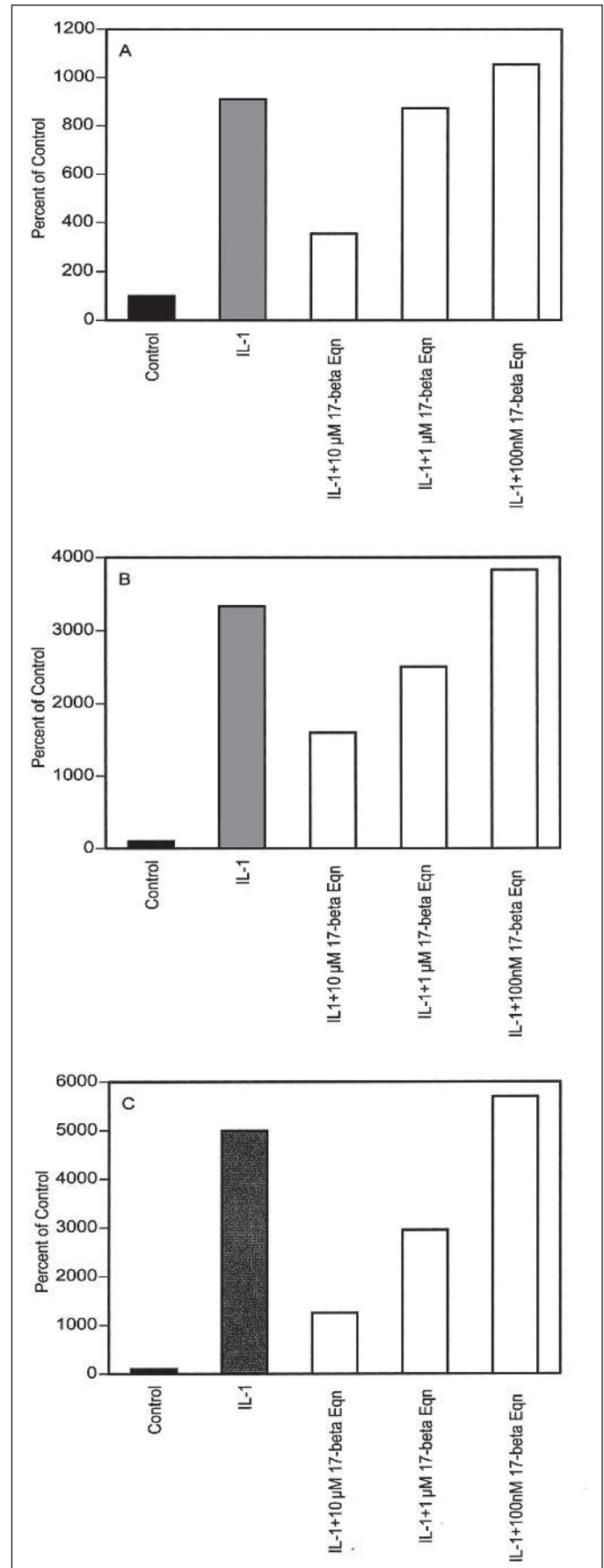
In order to determine a possible additive effect of 17 β -dihydroequilenin and 17 β -estradiol on IL-1 α -induced cytokine production in HUVECs, cells were treated with a combination of these two estrogens. As can be seen from Figure 5, when the cells were incubated with 1 μ M 17 β -dihydroequilenin in combination with 100nM 17 β -estradiol an additive effect in the reduction of IL-1 α -induced production of IL-6, IL-8 and MCP-1 was observed, resulting in a reduction of cytokine production below levels reached when HUVECs were incubated with each estrogen alone at the respective concentration. Such an effect was also seen for IL-8 and MCP-1 when either 100nM or 10nM 17 β -dihydroequilenin were combined with 100nM 17 β -estradiol.

The down-modulating effect of 17 β -dihydroequilenin (1 μ M) on IL-1 α -induced IL-6 protein production was almost completely reversed in the presence of 100-fold molar excess of the pure high affinity estrogen receptor antagonist ICI 182,780 (IL-1 α -induced IL-6 production: 274.3 \pm 37.4% of control; IL-1 α -induced IL-6 production in the presence of 17 β -dihydroequilenin: 184.5 \pm 37.4% of control; IL-1 α -induced IL-6 production in the presence of 17 β -dihydroequilenin and ICI 182,780: 251.1 \pm 62.3% of control). Similar results were obtained for IL-1 α -induced IL-8 and MCP-1 production. ICI 182,780 did not affect either basal or IL-1 α -induced expression of these inflammatory mediators (data not shown).

Effects of 17 β -dihydroequilenin on IL-1 α -induced IL-6, IL-8 and MCP-1 mRNA expression in HUVECs

As can be seen from Figure 6, IL-1 α significantly increased levels of mRNA specific for IL-6, IL-8 and MCP-1, respectively, in HUVECs. 17 β -dihydroequilenin used at a concentration of 1 μ M down regulated IL-1 α -induced mRNA levels for IL-8 and MCP-1 significantly, whereas a significant effect on IL-6 mRNA

Figure 6: Effects of 17 β -dihydroequilenin on IL-1 α -induced mRNA expression specific for IL-6 (panel A), IL-8 (panel B) and MCP-1 (panel C) in HUVECs: HUVECs were incubated for 24 hours without or with 17 β -dihydroequilenin at the indicated concentration. Thereafter human recombinant IL-1 α was added to the cells to give a final concentration of 200 U/ml and the cultures were incubated for a further 6 hours. mRNA was prepared and RealTime-PCR with primers specific for IL-6, IL-8, MCP-1 and GAPDH was performed as described in the *Methods* section. IL-6, IL-8 and MCP-1 mRNA levels were normalized according to the respective GAPDH mRNA levels. Experiments were performed two times. A representative experiment is shown.



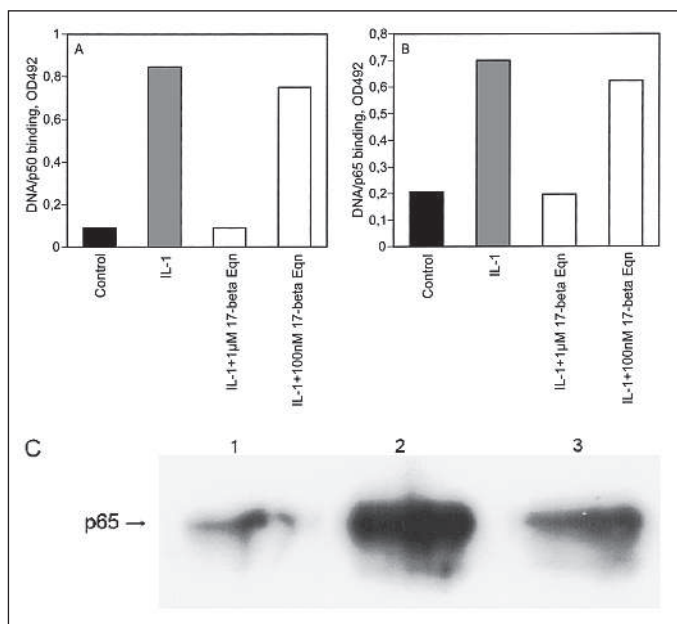


Figure 7: Effects of 17β-dihydroequilenin on IL-1α-induced NF-κB activation in HUVECs: HUVECs were incubated for 24 hours in 1.25% activated carbon absorbed FCS without or with 1 μM or 100 nM 17β-dihydroequilenin. Human recombinant IL-1α was added to such cells to give a final concentration of 200 U/ml and the cultures were incubated for a further 2 hours (panel A and B) or 30 minutes (panel C). Nuclear extracts were prepared from these cells and levels of p50 (panel A) and p65 (panel B) in these extracts were determined as described in the *Materials and Methods* section. Results of Western blotting with anti p65 antibodies are shown in panel C (lane 1: control; lane 2: IL-1α 200 U/ml; lane 3: 17β-dihydroequilenin 1μM+IL-1α 200 U/ml)

expression was seen at a concentration of 10μM. Similar to data obtained on the level of protein, 17α-dihydroequilenin, and δ-8,9-dehydroestrone down modulated this effect of IL-1α only at a concentration of 10μM whereas estrone had no significant effect on IL-1α-induced up regulation of IL-6, IL-8 and MCP-1 specific mRNA at any concentration used (data not shown).

Effects of 17β-dihydroequilenin on IL-1α-induced NF-κB activation in HUVECs

17β-dihydroequilenin at a concentration of 1μM reduced IL-1α-induced translocation of both p50 and p65 to the nucleus in HUVECs to control levels (Fig. 7A and B). The fact that 17β-dihydroequilenin at a concentration of 1μM blocks nuclear translocation of p65 was also confirmed by Western blot (Fig. 7C).

Discussion

We investigated here the anti-inflammatory potential of 17β-estradiol, estrone, which are the primary estrogen export products from the human ovary, and compared it to that of 17α-dihydroequilenin, 17β-dihydroequilenin, and δ-8,9-dehydroestrone in an *in vitro* model employing human endothelial cells activated with IL-1α. These estrogens are active components of Premarin which is commonly used to treat postmenopausal women (21). The concentrations of estrogens used in our experiments were

ranging from physiological to pharmacological and were comparable with concentrations used in others *in vitro* studies (12, 14). It should be mentioned, however, that pathophysiologically and physiologically relevant concentration *in vivo* are in the nanomolar rather than in the micromolar range. Under basal conditions human endothelial cells produced levels of IL-6, IL-8 and MCP-1 comparable to levels shown in other *in vitro* studies (25). As expected and as demonstrated before by others, IL-1α significantly increased the production of the inflammatory mediators IL-6, IL-8 and MCP-1 by human endothelial cells (26). However, 17β-dihydroequilenin, when added to the cells 24 hours before addition of IL-1α, counteracted the IL-1α-induced increase of IL-6, IL-8 and MCP-1 resulting in levels of these cytokines comparable to control levels in cells not treated with IL-1α. This effect was seen when 17β-dihydroequilenin and 17β-estradiol were used at a concentration of 1μM. Interestingly, when both compounds were used in combination at respective concentrations of 100nM a further reduction in IL-1α-induced IL-8 and MCP-1 production in endothelial cells was observed, suggesting an additive or cooperative effect of in our *in vitro* model. Such an additive effect was also seen when 17β-dihydroequilenin was used at a concentration of 10nM and combined with 100nM 17β-estradiol. In contrast, both 17α-dihydroequilenin and δ-8,9-dehydroestrone showed down regulation of IL-1α-induced production of IL-6, IL-8 and MCP-1 to a similar extent only when used at a concentration of 10μM whereas estrone had no effect. It should be mentioned that the metabolites tested in our study had no significant influence on basal, unstimulated production of the abovementioned mediators of inflammation in cultured human endothelial cells (data not shown).

Estrogens exert their effects either via rapid non-genomic or via long term regulations that involve changes in gene expression. Vasodilatation via increased NO synthesis is due to rapid non-genomic regulation. Genomic effects include inhibition of the response to vascular injury and preventive actions against atherosclerosis (27). Estrogens mediate their direct vascular effects via two ERs, namely ER-α and ER-β, which are members of the super family of steroid hormone receptors (28). A functional ER was identified in cultured human endothelial cells (29). The presence of only ER-α in HUVECs was also described (30). In contrast to this observation but in agreement with others we found both ER-α and ER-β protein and mRNA to be expressed by human endothelial cells using Western blotting and RT-PCR, respectively (31). It should be emphasized that the 17β-estradiol, an estrogen with highest ER binding affinity used in this study, was effective in counteracting the effects of IL-1α at a concentration of 100nM, followed by 17β-dihydroequilenin, which was effective in a tenfold lower concentration as 17α-dihydroequilenin and δ-8,9-dehydroestrone, which had been shown to have significantly lower binding affinities towards ERs (32). Surprisingly, estrone, which has a higher binding affinity than 17α-dihydroequilenin and δ-8,9-dehydroestrone, showed no effect (33).

In order to confirm that the effects of 17β-dihydroequilenin on IL-1α-mediated production of IL-6, IL-8 and MCP-1 involve changes in gene expression we performed RealTime-PCR. Again as expected, IL-1α significantly increased levels of

mRNA specific for IL-6, IL-8 and MCP-1, respectively, in endothelial cells. Similar to data obtained on the level of protein 17 β -dihydroequilenin down regulated IL-1 α -induced mRNA levels for these inflammatory mediators. 17 α -dihydroequilenin and δ -8,9-dehydroestrone showed such an effect only when used at a concentration of 10 μ M whereas estrone had no significant effect at any concentration used.

In our study 17 β -dihydroequilenin inhibition of IL-1 α -induced IL-6, IL-8 and MCP-1 protein production was partially abrogated in the presence of 100-fold molar excess of the pure high affinity estrogen receptor antagonist ICI 182,780. This suggests that the anti-inflammatory effect of 17 β -dihydroequilenin is mediated through one or both estrogen receptors, which are both expressed in endothelial cells used in this study. Similar to our observations, previous studies found that the inhibition of E-selectin and VCAM-1 expression by 17 β -estradiol and 17-epiestriol was significantly attenuated by ICI compounds in human endothelial cells (10, 12, 20)

Several groups have shown that NF- κ B and estrogen receptor signalling pathways interact (34). NF- κ B functions as a dimeric DNA-binding protein that comprises subunits from a family of related proteins called the Rel family of transcriptional activators including the mammalian proteins p50 and p65 (Rel A) (35). NF- κ B is known to mediate the transcriptional activation of a variety of cytokines including IL-6, IL-8 and MCP-1 (36). It was recently shown that 17 β -estradiol inhibited lipopolysaccharide (LPS)-induced activation of NF- κ B in human endothelial cells and IL-1 α -mediated activation of NF- κ B in rat brain endothelial cells (12, 37). 17-epiestriol, an estrogen metabolite, prevents tumor necrosis factor α -induced translocation of NF- κ B into the nucleus in HUVECs (20). In contrast to these observations, physiological concentrations of 17 β -estradiol were shown to activate NF- κ B within 10 minutes in human coronary artery endothelial cells (14). We found that 17 β -dihydroequilenin at a concentration of 1 μ M inhibited IL-1 α -induced translocation of p50 and p65 to the nucleus in IL-1 α -stimulated HUVECs indicating that the anti-inflammatory effect of 17 β -dihydroequilenin seen in our model seems to be mediated via interference with the NF- κ B-pathway.

In conclusion we have identified the estrogen metabolite 17 β -dihydroequilenin, a component of Premarin, which has been widely used in hormone replacement therapy in postmenopausal women, as an inhibitor of inflammatory activation of human endothelial cells. However, randomized hormone replacement therapy trials for primary and secondary prevention of cardiovascular disease found no evidence for beneficial and protective cardiovascular effects described in earlier studies but showed that hormone therapy does not delay the progression of atherosclerosis on the one hand, and does not decrease but rather increase the risk of coronary heart disease on the other hand (38). When considering these results it should be noted that also in primates estrogen treatment started after menopause did not show cardiovascular protection (39). Indeed in the Women's Health Initiative (WHI) trial due to the average age of 63 years of the women included in the study there was sufficient time past the onset of menopause for the development of atherosclerosis. In our study the cells were exposed to estrogen treatment prior to inflammatory stimulation with IL-1 α . In the light of contrasting results from epidemiological and experimental studies, it seems warranted to dissect possible beneficial or adverse effects of estrogens by studying the mode of action of various estrogen metabolites. This is especially important since hormone replacement therapy used in the clinical setting is in most cases based on a combination of various compounds. Thus, better characterization of specific estrogens or metabolites such as 17 β -dihydroequilenin could provide the basis for tailored therapies, which then might be able to achieve cardio- and vasoprotection without adverse side effects.

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