Modulation of estrogen signaling in hepatic and vascular tissue

Krom, Y.D.

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Reduced estrogen receptor alpha levels do not limit the anti-inflammatory effects of 17-beta-estradiol in endothelial cells

Krom Y.D[1,*], Carlotti F[2], Hoeben R.C[2], Frants R.R[1], Havekes L.M[3,4,5] and Willems van Dijk K[1,3]

[1]Department of Human Genetics, Leiden University Medical Center, The Netherlands
[2]Department of Molecular Cell Biology, Leiden University Medical Center, The Netherlands.
[3]Department of Endocrinology and Metabolic Diseases, Leiden University Medical Center, The Netherlands
[4]Department of Cardiology, Leiden University Medical Center, Leiden, The Netherlands
[5]TNO-Quality of Life, Gaubius Laboratory, Leiden, The Netherlands

Abstract

Objective: In the present study, the role of estrogen receptor alpha (ERα) in the anti-inflammatory effect of 17-β-estradiol (E_2) has been examined. ***Method:*** Endothelial cell lines with reduced ERα levels were generated by transduction with lentiviral vectors expressing short hairpin (sh)RNA constructs against ERα (shERα). Real time PCR was performed to quantify the expression levels of inflammatory cell adhesion molecules in stably transduced endothelial cells. ***Results:*** Expression levels of the adhesion molecules, E-selectin and intercellular adhesion molecule-1 (ICAM-1) were significantly induced by TNFα treatment, and were significantly inhibited by pre-treatment with E_2. Surprisingly, the shERα expressing endothelial cells, which displayed 50% reduced ERα mRNA levels and activity, responded in an identical manner to TNFα plus and minus E_2 pre-treatment. Complete abrogation of ERα activity, by supplementation of the antagonist ICI, however, did block the E_2 effect. ***Conclusion:*** ERα activity is required for the anti-inflammatory effect of E_2 but not in a "rate-limiting mode"

Introduction

Atherosclerosis is considered to be a chronic inflammatory process. One of the initial events involves the recruitment of inflammatory cells from the circulation into the developing lesion. This process is dependent on the expression of adhesion molecules such as E-selectin, vascular cellular adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1). Expression of adhesion molecules is increased in atherosclerotic lesions [1-5], whereas deficiency of these molecules has resulted in a reduction of atherosclerostic lesion size and number [6-9].

Atheroprotective properties of E_2 have been demonstrated in several animal models [10-12], however, the underlying mechanisms remain obscure. Adhesion molecules constitute a possible target for E_2, even though experimental and epidemiological studies have reported conflicting results. Some have reported that hormone replacement therapy in post-menopausal women with coronary artery disease results in a reduction of soluble adhesion molecules [13-15], while other studies did not report significant changes [16-18]. Also, in vitro studies, which measured the effect of E_2 on endothelial expression of adhesion molecules have reported both enhanced [19,20] and reduced [21-23] expression level of adhesion molecules.

The actions of E_2 are mainly exerted via estrogen receptors (ERs), which classically serve as ligand-activated transcription factors. To date two ERs, ERα and ERβ have been

identified [24-26]. Both ERα and ERβ are present in vascular endothelium [27], but their physiological roles are incompletely understood. In vitro studies indicate that both ERα and ERβ can mediate the anti-inflammatory effect of E_2 with respect to the expression of adhesion molecules [28,29]. Interestingly, ERα levels in atherosclerotic vessels have been documented to be lower as compared to their levels in normal vessels and vessels with a mild degree of atherosclerosis [30-32]. Whether the reduced level of functional ERα in endothelial cells results in a reduced response to E_2 and thus aggravates the atherosclerotic process is not known.

In the present study, we have evaluated the effect of E_2 on TNFα-induced expression of adhesion molecules in a mouse endothelial cell line. To gain insight into the biological role of ERα and the importance of ERα level in endothelial function, lentiviral vectors expressing short hairpin (sh)RNA targeted to ERα (shERα) were designed. Silencing of ERα gene expression as well as ERα functioning was established. However, in contrast to total ablation of ERα activity, 50% reduction in ERα activity did not affect the E_2 signaling cascade regarding down-regulation of TNFα-induced expression of adhesion molecules. Thus, in the current study we found that ERα is required but their levels are not rate-limiting in the anti-inflammatory response of E_2.

Results

Expression levels of adhesion molecules in mouse endothelial cells

Expression levels of several adhesion molecules were determined in a mouse endothelial cell line (H5V), both under basal as well as under stimulatory conditions. Whereas VCAM-1 levels were undetectable, ICAM-1 and E-selectin levels were expressed under basal conditions, with E-selectin showing the most abundant levels. TNFα treatment dose dependently induced the expression of ICAM-1, which leveled off at a dose of 100 units TNFα per well (Figure 1A).

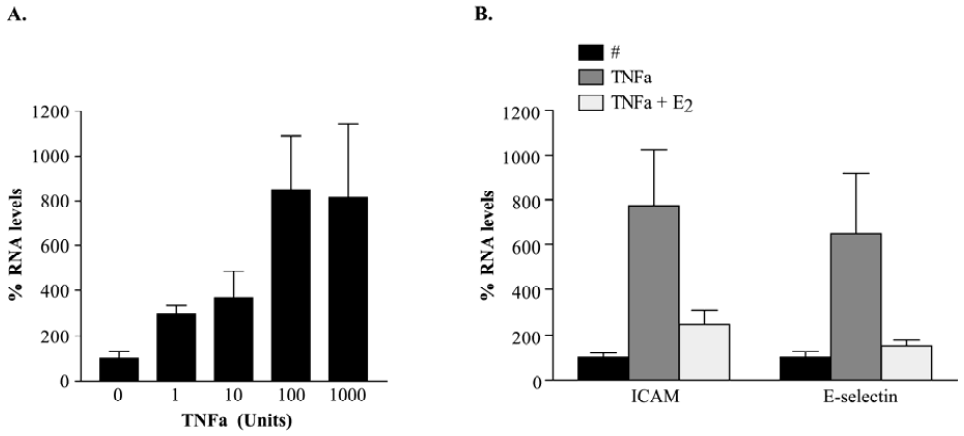

Figure 1. Expression of adhesion molecules in endothelial cells
(A) Endothelial cells were incubated with the indicated doses range of TNFα. Five hours after treatment RNA was extracted and subjected to taqman analysis to measure ICAM-1 levels. (B) The effect of E_2 on ICAM-1 and E-selectin expression in TNFα stimulated endothelial cells was assessed by taqman analysis. Dissolvent or 10^{-6}M E_2 was added 19 hours prior to TNFα (100 Units) treatment. The ratio of ICAM-1 / HPRT and E-selectin / HPRT of untreated cells was arbitrarily set as 100 for control. Data represented as mean ± SD.

E_2 and TNFα induced expression of adhesion molecules

As depicted in figure 1B, pretreatment of H5V cells with E_2 (10^{-8}M, 24 hours) significantly diminished the TNFα-mediated increase in both ICAM-1 and E-selectin levels. ICAM-1 levels were reduced from 774 ± 248% to 246 ± 61% and E-selectin levels from 650 ± 270% to 153 ± 24% ($P<0.05$). In a another endothelial cell line, hemangioendothelioma-derived cells (EOMAs), we were able to reproduce the repressive effect of E_2 (data not shown).

ERα levels and activity in shERα expressing endothelial cells

To knockdown the endogenously expressed ERα, a near 100% stable shERα expressing endothelial cell line was generated by selecting for GFP positive cells. RNA was isolated from Lenti-Empty (control) and Lenti-shERα cells 24 hours after E_2 (10^{-8}M) treatment to evaluate the silencing effect. As depicted in Fig 2A, real-time PCR analysis demonstrated up to 50% reduced ERα RNA levels in the shERα expressing H5V cells. In control as well as shERα expressing endothelial cells, ERα RNA levels were not modified upon E_2 and ICI treatment (data not shown)

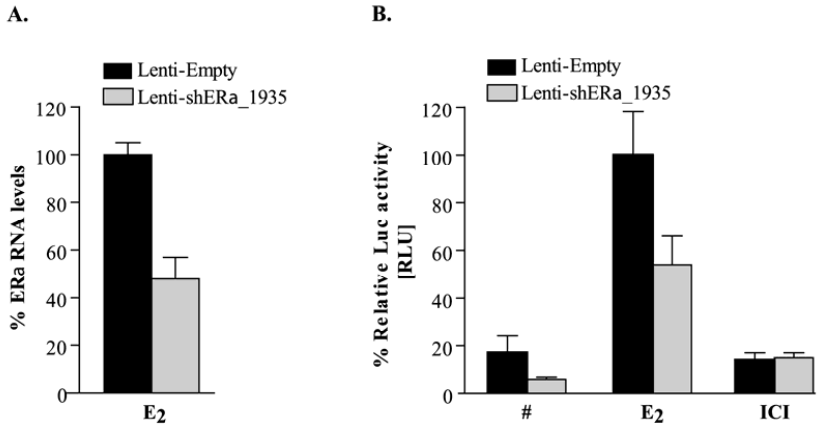

Figure 2. Mouse ERα mRNA and activity in stable shERα expressing endothelial cells
(A) RNA was extracted from H5V cells infected either with Lenti_Empty or Lenti_shERα constructs. ERα levels were assessed by taqman analysis. HPRT was used as internal standard. Data represented as mean ± SD. (B) Both Lenti_Empty and Lenti_shERα transduced H5V cells were co-transfected with pERE-Luc and pCMV-LacZ. The cells were stimulated with 10^{-8}M E_2 or 10^{-8}M E_2 plus 10^{-6}M ICI for 24 hours. Luciferase activity was measured 48 hours after transfection. Data represented as mean ± SD. E_2 induced luciferase activity in Lenti_Empty cells was arbitrarily set as 100%.

Subsequently, the effect of reduced ERα levels on ERα mediated transcription was evaluated. To this end, cells were transfected with a reporter plasmid carrying the estrogen response element upstream of the luciferase gene (pERE-Luc) and were treated with either dissolvent, E_2 or E_2 + ICI. As a result, control H5V cells showed enhanced ERα activity upon E_2 treatment, which was totally abolish by ICI treatment (Fig 2B). On the other hand, introduction of shERα led to a significant repression of ERα activity, as reflected by a 64% reduction under basal conditions and 46% reduction in the E_2 treated cells. Thus, shERα expressing lentiviral vectors significantly reduced ERα RNA levels and suppressed ERα mediated transcription.

ERα Knockdown in H5V cells and response to TNFα and E_2

The ERα knockdown H5V cells were used to explore the role of ERα in the E_2-induced repression of E-selectin and ICAM-1 expression. As shown in Fig 3, also in the ERα knock-down cell line, TNFα induces the expression of E-selectin and ICAM-1 levels. Though, reduced ERα levels, E_2 was able to significantly reduce the TNFα induced effect. On the

other hand, the antagonist ICI, which has been shown to silence ERα activity completely (Fig 2B), did abrogate the E_2 effect on the expression of adhesion molecules (Fig 3). Thus, while complete abolishment of ERα activity did abort the repressive effect of E_2 on adhesion molecule, reduced ERα activity did not.

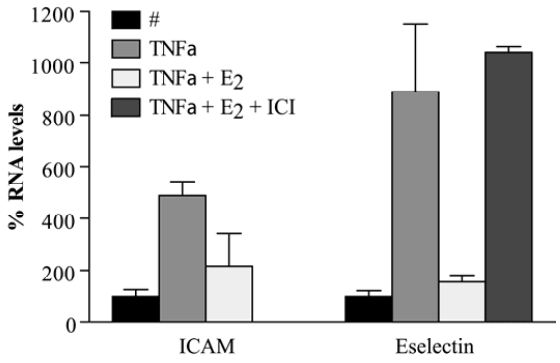

Figure 3. Expression of adhesion molecules in shERα expressing endothelial cells
Either dissolvent, 10^{-6}M E_2 or 10^{-6}M E_2 plus 10^{-6}M ICI was added 19 hours prior to TNFα (100 Units) treatment in Lenti_shERα transduced endothelial cells. Five hours after treatment RNA was extracted and subjected to taqman analysis to measure ICAM-1 levels and E-selectin levels. The ratio of ICAM-1 / HPRT and E-selectin / HPRT of untreated cells was arbitrarily set as 100 for control. Data represented as mean ± SD.

Discussion

Our results demonstrate that E_2 inhibits TNFα-induced expression of ICAM-1 and E-selectin levels in endothelial cells. To determine the role of ERα activity in this process, a stable shERα expressing endothelial cell line was generated. This shERα expressing cell line contained 50% reduced ERα mRNA levels resulting in 50% decreased ERα activity. Repression of endogenously expressed ERα, however, did not affect the E_2 inhibitory effect on expression of endothelial adhesion molecules. In contrast, complete silencing of ERα activity by use of the antagonist ICI did efficiently reverse the E_2 effect. Apparently, ERα activity is required for the anti-inflammatory response of E_2 with regard to inhibition of adhesion molecules, but the number of ERα molecules and level of ERα activity does not limit this response.

The observation that pre-treatment with E_2 significantly reduces the cytokine-induced expression of the endothelial adhesion molecules E-selectin and ICAM-1, implicates that E_2 makes the endothelium less responsive to the inflammatory microenvironment. In vitro studies with opposite results have also been published [19,20]. In those studies, E_2 was added simultaneously with the cytokine instead of before treatment as applied here, which could explain the discrepancy. Remarkably, in animal models beneficial effects were only observed

if E_2 was administrated prior to the development of atherosclerosis and not when arterial damage was present prior to hormone treatment [33-36]. From these studies we hypothesize that E_2 prevents atherosclerosis by interfering either prior to injury or very early post-injury.

RT-PCR analysis revealed that the TNFα-induced mRNA expression levels of E-selectin and ICAM-1 are reduced by E_2, indicating that the down regulation occurs at the transcriptional level. Since the ER antagonist ICI blocks the inhibitory effect of E_2 and the endothelial cells used in the current paper express only ERα and not ERβ, ERα seems to be involved. ERs are classically identified as ligand dependent transcription factors. The 5'regulatory regions of E-selectin and ICAM-1 do not contain classical estrogen response element (ERE) sites. Therefore it seems likely that gene transcription is affected by "cross-talk" of ERα with other transcription factors, such as Nf-kB, which is required for TNFα-mediated gene activation and which acts upon a specific site in the 5'regulatory regions of both E-selectin and ICAM-1. Previous studies have reported that E_2 could inhibit nuclear translocation and DNA binding of NF-κB [23] and that ERα could reduce the expression of an NF-κB-driven reporter plasmid [28]. In addition, a human E-selectin promoter study has revealed that the NF-κB site is required for the repressive effect of E_2 [29]. Thus, ERα is likely involved via a non-classical transcription pathway in the E_2 mediated inhibition of TNFα-induced E-selectin and ICAM-1 expression.

To address the question whether the level of ERα is limiting the effect of E_2 in modulating the TNFα response, we decreased endogenously expressed ERα RNA levels in endothelial cells by lentiviral-mediated expression of shERα. Due to integration of the transgene into the genome, silencing was maintained during at least 18 weeks of continuous culturing (data not shown). The 50% knockdown of ERα RNA levels, which coincided with ~50% repression of E_2 induced reporter gene expression did not change the E_2 mediated response towards the expression of adhesion factors. Since ICI blocks the E_2 effect, it is not likely that the E_2 mediated reduction is obtained through an ERα-independent pathway. Probably, the remaining 50% ERα activity is sufficient to inhibit expression of adhesion molecules. Thus, apparently fluctuating ERα levels in endothelial cells do not modulate the responsiveness with regard to E_2 mediated regulation of adhesion molecules. It will be of interest to address the role of ERβ in this process.

In summary, our findings suggest that E_2 has anti-inflammatory properties, as it could down-regulate E-selectin and ICAM-1 expression in endothelial cells. We found that ERα is required, but absolute ERα levels do not determine this anti-inflammatory effect.

Methods

Cell Culture

H5V (a murine endothelial cell line derived from heart) and EOMA (murine hemangioma-derived micro vascular cell line) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% fetal calf serum (FCS), 100 units/ml Penicillin, 100 μg/ml Streptomycin and glutamax (Invitrogen) (Complete DMEM). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. In experimental setting to reduce basal E_2 effects, H5V cells were switched to DMEM without phenol red (Gibco BRL) supplemented with 10% charcoal treated FCS (charcoal (Merck) 5gr/ 50ml FCS, mix overnight 4 degrees, centrifuge and filter-sterilize), 100 units/ml Penicillin, 100 μg/ml Streptomycin and glutamax (Invitrogen).

Adhesion experiment

24 hours before treatment, $1{,}5.10^5$ cells were seeded in triplicate in 12-wells plate. Either dissolvant, 10^{-8}M E_2 or 10^{-8}M E_2 + 10^{-6}M ICI was added for an additional 24 hours. 5 hours prior to RNA isolation, the indicated amount of TNFα (GF027, Chemicon) was added (0-1.000 Units). RNA was extracted by 250 μl/well Trizol.

Production of recombinant lentiviruses

The vectors used in our study are so-called SIN vectors, which lose the activity of the promoter located in the 5VLTR upon replication and integration into the genome of the host cells. The Rev-responsive element sequence is recognized by the viral Rev protein and is essential to regulate the production of viral mRNA [37]. The central polypurine tract (cPPT), which is located in the pol region of HIV-1, is retained in the vector as it has been reported to increase nuclear transport of the virus preintegration complex and hence increase transduction efficiency [38,39]. The PRE (posttranscriptional regulatory element) from the human hepatitis B virus (HBV) is a cis-acting sequence that increases expression of transgenes probably by stimulating nuclear export of the mRNA [40]. Expression of the transgene is under the control of an internal promoter: this will be the only mRNA transcribed. The vector plasmids were all derivatives of the pRRL-cPPT-X-PRE-SIN [40]. Plasmids pRRL-cPPT-CMV-GFP-PRE-SIN (here named pLenti-Empty), pRRL-H1 promoter-shERα_1395-cPPT-CMV-GFP-PRE-SIN

(here named pLenti-shERα) and pRRL-H1 promoter-shERα_1103-cPPT-CMV-GFP-PRE-SIN (here named pLenti-shERα_1103) (Figure 3) were constructed with a cytomegalovirus promoter driving the green fluorescent protein. The vectors were produced as described previously [41]. Briefly, the lentiviral backbone containing the gene of interest and the three ''helper'' plasmids (encoding HIV-1 gag – pol, HIV-1 rev, and VSV-G envelope) were cotransfected overnight using the calcium phosphate method into 293T cells. The medium was refreshed and viruses were harvested after 48 and 72 h, passed through 0.45-Am filters, and stored at -80ºC. Virus was quantitated by antigen capture ELISA measuring HIV p24 levels (ZeptoMetrix Corp., New York, NY, USA) as described [42].

Lentivirus transduction

24 hours before infection, cells were seeded into 96 wells plates (Greiner). H5V at $1{,}5.10^3$ cells/well and EOMA 4.10^3 cells/well. Viral supernatants were added to fresh medium supplemented with 8 µg/ml Polybrene (Sigma), and the cells were incubated overnight. The next day, the medium was replaced with fresh medium. Transduction efficiency was analyzed 3 to 6 days post transduction by FACs analysis. HIV-1 reverse transcriptase inhibitor AZT (GlaxoWellcome) was added to transduced cells at a final concentration of 20 µg/ml.

FACS analysis

For FACS analyses, H5V and EOMA cells were trypsinized gently, the volume was increased by adding PBS/1% FCS, and the cells were kept on ice. The samples were analyzed with a FACScan flow cytometer (Becton – Dick-inson). GFP fluorescence was detected using a 530/30 nm bandpass filter (FL1 channel) following excitation with an argon ion laser source at 488 nm. Using a forward-scatter/side-scatter representation of events, a region was defined to exclude cellular debris from the analysis. A number of events/FL1 (which reflects the fluorescence intensity) histogram was then established according to this region, and percentages of GFP-positive cells were determined in comparison to the negative control (untreated cells). Data analysis was performed using CellQuest 3.1 software (Becton – Dick-inson). For each sample, 10.000 events were collected.

Select GFP positive cells to obtain 100% GFP expressing cell population

EOMA and H5V cells were exposed to shERα expressing lentiviral vectors at MOI 20 and tracking of GFP positive cells monitored the efficiency of gene transfer. Three days post-

infection, a maximum of 50% GFP positive cells was detected. As higher MOI appeared to be toxic, GFP positive cells of the Lenti-Empty, Lenti-shERα transduced population were diluted over 96-wells plate. FACS analysis was performed to select for and pool the cell populations containing near 100% GFP positive cells that consist of the same transgen.

Table 1. Primer sequences of genes used for mRNA quantification

Gene	*Forward primer*	*Reverse primer*
mERα	5'-CTAGCAGATAGGGAGCTGGTTCA	5'-GGAGATTCAAGTCCCCAAAGC
mERβ	5'- TCCTGATGCTTCTTTCTCATGTCA	5'-CACTTCATGCTGAGCAGATGTTC
E-selectin	5'- CCCTGCCCACGGTATCAG	5'-CCCTTCCACACAGTCAAACGT
ICAM-1	5'- GGACCACGGAGCCAATTTC	5'-CTCGGAGACATTAGAGAACAATGC
VCAM-1	5'- ACAAAACGATCGCTCAAATCG	5'-CGCGTTTAGTGGGCTGTCTATC

Real time quantitative PCR analysis

Total RNA was extracted from cells using TRIzol reagent (Life technologies). Purified RNA was treated with RQ1 RNase-free DNase (Promega, 1 units/ 2 μg of total RNA) and reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative gene expression analysis was performed on an ABI prism7700 Sequence Detection System (Applied Biosystems) using SYBR Green as described earlier [43]. PCR primer sets (TABLE I) were designed via Primer Express 1.7 software with the manufacturer's default settings (Applied Biosystems) and were validated for amplification efficiency. The absence of genomic DNA contamination in the RNA preparations was confirmed in a separate PCR reaction on total RNA samples that were not reverse transcribed. HPRT was used as the standard housekeeping gene. The significance of differences in relative gene expression numbers C_t ($C_{t(HPRT)}$–$C_{t(target\ gene)}$) measured by real time quantitative PCR was calculated using a Mann-Whitney U test. Probability values less than 0.05 were considered significant.

Luciferase reporter assay

Transient transfections were performed in triplicate in 12-wells plates ($1{,}5.10^5$ cells per well) using Lipofectamine (Invitrogen). The effect of lenti-shERα on ERα mediated transcription regulation was determined by co-transfecting the cells with 150ng of reporter construct $(ERE)_3$TATA-LUC and 300 ng pCMV-LacZ. After 24 hours, the cells were stimulated with complete DMEM containing 10^{-8}M E_2 or 10^{-8}M E_2 + 10^{-6}M ICI for an additional 24 hours. The cells were lysed with reporter lyses buffer (Promega) and after centrifugation of 2 min, supernatant was used for determining β-galactosidase normalized luciferase activity by adding 100 µl luciferyl-CoA (Promega) to 20 µl of cell extract in a monolight luminometer (BD Biosciences). β-galactosidase was measured in a 96-well microtiter plate using the β-Galactosidase Enzyme Assay System in reporter lyses buffer (Promega). Absorbance at 450 nm was determined in a microplate reader. Luciferase activities were normalized for transfection efficiency with the β-galactosidase activity and expressed as a percentage relative to expression levels induced by endogenous estrogen receptor (ER). Expression of endogenous ERα was verified by real time PCR.

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