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Modulation of estrogen signaling in hepatic and vascular tissue

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Citation

Krom, Y. D. (2006, November 7). *Modulation of estrogen signaling in hepatic and vascular tissue*. Retrieved from <https://hdl.handle.net/1887/4967>

Version: Corrected Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).

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

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Abstract

Objective: In the present study, the role of estrogen receptor alpha (ER α) in the anti-inflammatory effect of 17- β -estradiol (E₂) has been examined. **Method:** Endothelial cell lines with reduced ER α levels were generated by transduction with lentiviral vectors expressing short hairpin (sh)RNA constructs against ER α (shER α). 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 TNF α treatment, and were significantly inhibited by pre-treatment with E₂. Surprisingly, the shER α expressing endothelial cells, which displayed 50% reduced ER α mRNA levels and activity, responded in an identical manner to TNF α plus and minus E₂ pre-treatment. Complete abrogation of ER α activity, by supplementation of the antagonist ICI, however, did block the E₂ effect. **Conclusion:** ER α activity is required for the anti-inflammatory effect of E₂ 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 atherosclerotic lesion size and number [6-9].

Atheroprotective properties of E₂ have been demonstrated in several animal models [10-12], however, the underlying mechanisms remain obscure. Adhesion molecules constitute a possible target for E₂, 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 E₂ on endothelial expression of adhesion molecules have reported both enhanced [19,20] and reduced [21-23] expression level of adhesion molecules.

The actions of E₂ are mainly exerted via estrogen receptors (ERs), which classically serve as ligand-activated transcription factors. To date two ERs, ER α and ER β have been

identified [24-26]. Both ER α and ER β are present in vascular endothelium [27], but their physiological roles are incompletely understood. In vitro studies indicate that both ER α and ER β can mediate the anti-inflammatory effect of E₂ with respect to the expression of adhesion molecules [28,29]. Interestingly, ER α 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 ER α in endothelial cells results in a reduced response to E₂ and thus aggravates the atherosclerotic process is not known.

In the present study, we have evaluated the effect of E₂ on TNF α -induced expression of adhesion molecules in a mouse endothelial cell line. To gain insight into the biological role of ER α and the importance of ER α level in endothelial function, lentiviral vectors expressing short hairpin (sh)RNA targeted to ER α (shER α) were designed. Silencing of ER α gene expression as well as ER α functioning was established. However, in contrast to total ablation of ER α activity, 50% reduction in ER α activity did not affect the E₂ signaling cascade regarding down-regulation of TNF α -induced expression of adhesion molecules. Thus, in the current study we found that ER α is required but their levels are not rate-limiting in the anti-inflammatory response of E₂.

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. TNF α treatment dose dependently induced the expression of ICAM-1, which leveled off at a dose of 100 units TNF α per well (Figure 1A).

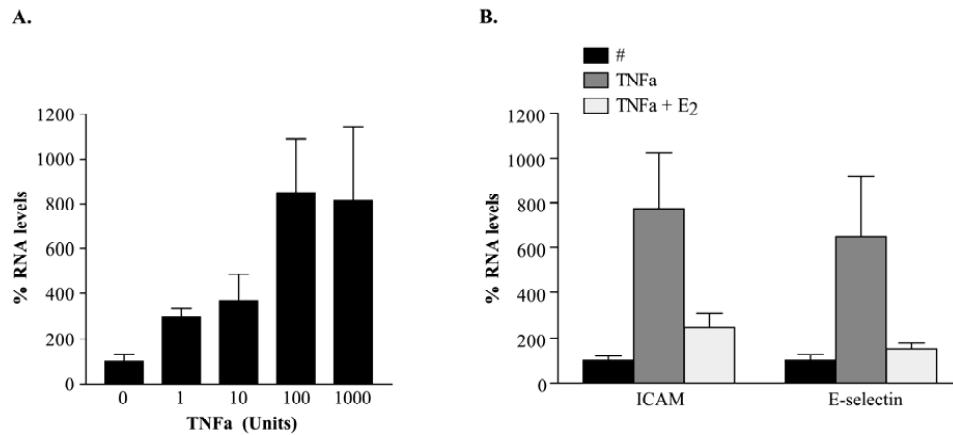


Figure 1. Expression of adhesion molecules in endothelial cells

(A) Endothelial cells were incubated with the indicated doses range of TNF α . Five hours after treatment RNA was extracted and subjected to taqman analysis to measure ICAM-1 levels. (B) The effect of E₂ on ICAM-1 and E-selectin expression in TNF α stimulated endothelial cells was assessed by taqman analysis. Dissolvent or 10⁻⁶M E₂ was added 19 hours prior to TNF α (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 \pm SD.

E₂ and TNF α induced expression of adhesion molecules

As depicted in figure 1B, pretreatment of H5V cells with E₂ (10⁻⁸M, 24 hours) significantly diminished the TNF α -mediated increase in both ICAM-1 and E-selectin levels. ICAM-1 levels were reduced from 774 \pm 248% to 246 \pm 61% and E-selectin levels from 650 \pm 270% to 153 \pm 24% (P<0.05). In a another endothelial cell line, hemangioendothelioma-derived cells (EOMAs), we were able to reproduce the repressive effect of E₂ (data not shown).

ER α levels and activity in shER α expressing endothelial cells

To knockdown the endogenously expressed ER α , a near 100% stable shER α expressing endothelial cell line was generated by selecting for GFP positive cells. RNA was isolated from Lenti-Empty (control) and Lenti-shER α cells 24 hours after E₂ (10⁻⁸M) treatment to evaluate the silencing effect. As depicted in Fig 2A, real-time PCR analysis demonstrated up to 50% reduced ER α RNA levels in the shER α expressing H5V cells. In control as well as shER α expressing endothelial cells, ER α RNA levels were not modified upon E₂ and ICI treatment (data not shown)

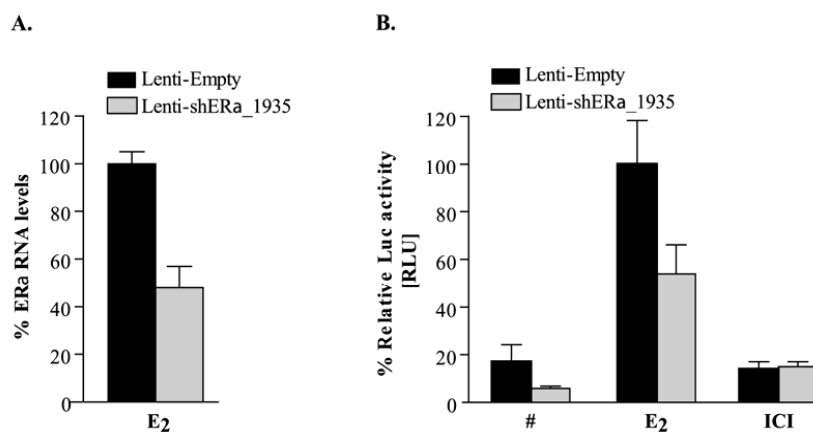


Figure 2. Mouse ER α mRNA and activity in stable shER α expressing endothelial cells

(A) RNA was extracted from H5V cells infected either with Lenti_Empty or Lenti_shER α constructs. ER α levels were assessed by taqman analysis. HPRT was used as internal standard. Data represented as mean \pm SD. (B) Both Lenti_Empty and Lenti_shER α transduced H5V cells were co-transfected with pERE-Luc and pCMV-LacZ. The cells were stimulated with 10^{-8} M E₂ or 10^{-8} M E₂ plus 10^{-6} M ICI for 24 hours. Luciferase activity was measured 48 hours after transfection. Data represented as mean \pm SD. E₂ induced luciferase activity in Lenti_Empty cells was arbitrarily set as 100%.

Subsequently, the effect of reduced ER α levels on ER α 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, E₂ or E₂ + ICI. As a result, control H5V cells showed enhanced ER α activity upon E₂ treatment, which was totally abolish by ICI treatment (Fig 2B). On the other hand, introduction of shER α led to a significant repression of ER α activity, as reflected by a 64% reduction under basal conditions and 46% reduction in the E₂ treated cells. Thus, shER α expressing lentiviral vectors significantly reduced ER α RNA levels and suppressed ER α mediated transcription.

ER α Knockdown in H5V cells and response to TNF α and E₂

The ER α knockdown H5V cells were used to explore the role of ER α in the E₂-induced repression of E-selectin and ICAM-1 expression. As shown in Fig 3, also in the ER α knock-down cell line, TNF α induces the expression of E-selectin and ICAM-1 levels. Though, reduced ER α levels, E₂ was able to significantly reduce the TNF α induced effect. On the

other hand, the antagonist ICI, which has been shown to silence ER α activity completely (Fig 2B), did abrogate the E₂ effect on the expression of adhesion molecules (Fig 3). Thus, while complete abolishment of ER α activity did abort the repressive effect of E₂ on adhesion molecule, reduced ER α activity did not.

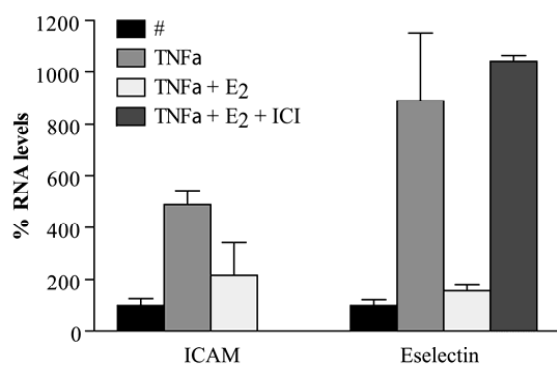


Figure 3. Expression of adhesion molecules in shER α expressing endothelial cells

Either dissolvent, 10⁻⁶M E₂ or 10⁻⁶M E₂ plus 10⁻⁶M ICI was added 19 hours prior to TNF α (100 Units) treatment in Lenti_shER α 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 \pm SD.

Discussion

Our results demonstrate that E₂ inhibits TNF α -induced expression of ICAM-1 and E-selectin levels in endothelial cells. To determine the role of ER α activity in this process, a stable shER α expressing endothelial cell line was generated. This shER α expressing cell line contained 50% reduced ER α mRNA levels resulting in 50% decreased ER α activity. Repression of endogenously expressed ER α , however, did not affect the E₂ inhibitory effect on expression of endothelial adhesion molecules. In contrast, complete silencing of ER α activity by use of the antagonist ICI did efficiently reverse the E₂ effect. Apparently, ER α activity is required for the anti-inflammatory response of E₂ with regard to inhibition of adhesion molecules, but the number of ER α molecules and level of ER α activity does not limit this response.

The observation that pre-treatment with E₂ significantly reduces the cytokine-induced expression of the endothelial adhesion molecules E-selectin and ICAM-1, implicates that E₂ makes the endothelium less responsive to the inflammatory microenvironment. In vitro studies with opposite results have also been published [19,20]. In those studies, E₂ 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 E₂ 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 E₂ prevents atherosclerosis by interfering either prior to injury or very early post-injury.

RT-PCR analysis revealed that the TNF α -induced mRNA expression levels of E-selectin and ICAM-1 are reduced by E₂, indicating that the down regulation occurs at the transcriptional level. Since the ER antagonist ICI blocks the inhibitory effect of E₂ and the endothelial cells used in the current paper express only ER α and not ER β , ER α 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 ER α with other transcription factors, such as NF- κ B, which is required for TNF α -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 E₂ could inhibit nuclear translocation and DNA binding of NF- κ B [23] and that ER α could reduce the expression of an NF- κ B-driven reporter plasmid [28]. In addition, a human E-selectin promoter study has revealed that the NF- κ B site is required for the repressive effect of E₂ [29]. Thus, ER α is likely involved via a non-classical transcription pathway in the E₂ mediated inhibition of TNF α -induced E-selectin and ICAM-1 expression.

To address the question whether the level of ER α is limiting the effect of E₂ in modulating the TNF α response, we decreased endogenously expressed ER α RNA levels in endothelial cells by lentiviral-mediated expression of shER α . 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 ER α RNA levels, which coincided with ~50% repression of E₂ induced reporter gene expression did not change the E₂ mediated response towards the expression of adhesion factors. Since ICI blocks the E₂ effect, it is not likely that the E₂ mediated reduction is obtained through an ER α -independent pathway. Probably, the remaining 50% ER α activity is sufficient to inhibit expression of adhesion molecules. Thus, apparently fluctuating ER α levels in endothelial cells do not modulate the responsiveness with regard to E₂ mediated regulation of adhesion molecules. It will be of interest to address the role of ER β in this process.

In summary, our findings suggest that E₂ has anti-inflammatory properties, as it could down-regulate E-selectin and ICAM-1 expression in endothelial cells. We found that ER α is required, but absolute ER α 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 μ g/ml Streptomycin and glutamax (Invitrogen) (Complete DMEM). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. In experimental setting to reduce basal E₂ 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 μ g/ml Streptomycin and glutamax (Invitrogen).

Adhesion experiment

24 hours before treatment, $1.5 \cdot 10^5$ cells were seeded in triplicate in 12-wells plate. Either dissolvant, 10^{-8} M E₂ or 10^{-8} M E₂ + 10^{-6} M ICI was added for an additional 24 hours. 5 hours prior to RNA isolation, the indicated amount of TNF α (GF027, Chemicon) was added (0-1.000 Units). RNA was extracted by 250 μ l/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 5'LTR 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-shER α _1395-cPPT-CMV-GFP-PRE-SIN

(here named pLenti-shER α) and pRRL-H1 promoter-shER α _1103-cPPT-CMV-GFP-PRE-SIN (here named pLenti-shER α _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- μ m filters, and stored at -80°C. 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 $1.5 \cdot 10^3$ cells/well and EOMA $4 \cdot 10^3$ cells/well. Viral supernatants were added to fresh medium supplemented with 8 μ g/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 μ g/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 – Dickinson). 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 – Dickinson). 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 shER α 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-shER α 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

<i>Gene</i>	<i>Forward primer</i>	<i>Reverse primer</i>
mER α	5'-CTAGCAGATAGGGAGCTGGTTCA	5'-GGAGATTCAAGTCCCCAAAGC
mER β	5'- TCCTGATGCTTCTTTCTCATGTCA	5'-CACTTCATGCTGAGCAGATGTTT
E-selectin	5'- CCCTGCCCACGGTATCAG	5'-CCCTCCACACAGTCAAACGT
ICAM-1	5'- GGACCACGGAGCCAATTC	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 μ g 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 ($1.5 \cdot 10^5$ cells per well) using Lipofectamine (Invitrogen). The effect of lenti-shER α on ER α mediated transcription regulation was determined by co-transfecting the cells with 150ng of reporter construct (ERE)₃TATA-LUC and 300 ng pCMV-LacZ. After 24 hours, the cells were stimulated with complete DMEM containing 10^{-8} M E₂ or 10^{-8} M E₂ + 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 μ l luciferyl-CoA (Promega) to 20 μ l 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 ER α was verified by real time PCR.

References

1. Nakashima Y, Raines EW, Plump AS, Breslow JL, Ross R: **Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse.** *Arterioscler Thromb Vasc Biol* 1998, **18**: 842-851.
2. Richardson M, Hadcock SJ, DeReske M, Cybulsky MI: **Increased expression in vivo of VCAM-1 and E-selectin by the aortic endothelium of normolipemic and hyperlipemic diabetic rabbits.** *Arterioscler Thromb* 1994, **14**: 760-769.
3. Johnson-Tidey RR, McGregor JL, Taylor PR, Poston RN: **Increase in the adhesion molecule P-selectin in endothelium overlying atherosclerotic plaques. Coexpression with intercellular adhesion molecule-1.** *Am J Pathol* 1994, **144**: 952-961.
4. Walpolo PL, Gotlieb AI, Cybulsky MI, Langille BL: **Expression of ICAM-1 and VCAM-1 and monocyte adherence in arteries exposed to altered shear stress.** *Arterioscler Thromb Vasc Biol* 1995, **15**: 2-10.
5. Iiyama K, Hajra L, Iiyama M, Li H, DiChiara M, Medoff BD, Cybulsky MI: **Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation.** *Circ Res* 1999, **85**: 199-207.
6. Bourdillon MC, Poston RN, Covacho C, Chignier E, Bricca G, McGregor JL: **ICAM-1 deficiency reduces atherosclerotic lesions in double-knockout mice (ApoE(-/-)/ICAM-1(-/-)) fed a fat or a chow diet.** *Arterioscler Thromb Vasc Biol* 2000, **20**: 2630-2635.

7. Collins RG, Velji R, Guevara NV, Hicks MJ, Chan L, Beaudet AL: **P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice.** *J Exp Med* 2000, **191**: 189-194.
8. Dong ZM, Chapman SM, Brown AA, Frenette PS, Hynes RO, Wagner DD: **The combined role of P- and E-selectins in atherosclerosis.** *J Clin Invest* 1998, **102**: 145-152.
9. Nageh MF, Sandberg ET, Marotti KR, Lin AH, Melchior EP, Bullard DC, Beaudet AL: **Deficiency of inflammatory cell adhesion molecules protects against atherosclerosis in mice.** *Arterioscler Thromb Vasc Biol* 1997, **17**: 1517-1520.
10. Bourassa PA, Milos PM, Gaynor BJ, Breslow JL, Aiello RJ: **Estrogen reduces atherosclerotic lesion development in apolipoprotein E-deficient mice.** *Proc Natl Acad Sci U S A* 1996, **93**: 10022-10027.
11. Hodgins JB, Krege JH, Reddick RL, Korach KS, Smithies O, Maeda N: **Estrogen receptor alpha is a major mediator of 17beta-estradiol's atheroprotective effects on lesion size in Apoe^{-/-} mice.** *J Clin Invest* 2001, **107**: 333-340.
12. Marsh MM, Walker VR, Curtiss LK, Banka CL: **Protection against atherosclerosis by estrogen is independent of plasma cholesterol levels in LDL receptor-deficient mice.** *J Lipid Res* 1999, **40**: 893-900.
13. Caulin-Glaser T, Farrell WJ, Pfau SE, Zaret B, Bunger K, Setaro JF, Brennan JJ, Bender JR, Cleman MW, Cabin HS, Remetz MS: **Modulation of circulating cellular adhesion molecules in postmenopausal women with coronary artery disease.** *J Am Coll Cardiol* 1998, **31**: 1555-1560.
14. Kennedy G, McLaren M, Belch JJ, Seed M: **Elevated levels of sE-selectin in postmenopausal females are decreased by hormone replacement therapy to levels observed in pre-menopausal females.** *Thromb Haemost* 1999, **82**: 1433-1436.
15. Koh KK, Cardillo C, Bui MN, Hathaway L, Csako G, Waclawiw MA, Panza JA, Cannon RO, III: **Vascular effects of estrogen and cholesterol-lowering therapies in hypercholesterolemic postmenopausal women.** *Circulation* 1999, **99**: 354-360.
16. Jilka B, Eichler HG, Breiteneder H, Wolzt M, Aringer M, Graninger W, Rohrer C, Veitl M, Wagner OF: **Effects of 17 beta-estradiol on circulating adhesion molecules.** *J Clin Endocrinol Metab* 1994, **79**: 1619-1624.
17. Koh KK, Bui MN, Mincemoyer R, Cannon RO, III: **Effects of hormone therapy on inflammatory cell adhesion molecules in postmenopausal healthy women.** *Am J Cardiol* 1997, **80**: 1505-1507.
18. Oger E, Alhenc-Gelas M, Plu-Bureau, Mennen L, Cambillau M, Guize L, Pujol Y, Scarabin P: **Association of circulating cellular adhesion molecules with menopausal status and hormone replacement therapy. Time-dependent change in transdermal, but not oral estrogen users.** *Thromb Res* 2001, **101**: 35-43.
19. Cid MC, Kleinman HK, Grant DS, Schnaper HW, Fauci AS, Hoffman GS: **Estradiol enhances leukocyte binding to tumor necrosis factor (TNF)-stimulated endothelial cells via an increase in TNF-induced adhesion molecules E-selectin, intercellular adhesion molecule type 1, and vascular cell adhesion molecule type 1.** *J Clin Invest* 1994, **93**: 17-25.
20. Zhang X, Wang LY, Jiang TY, Zhang HP, Dou Y, Zhao JH, Zhao H, Qiao ZD, Qiao JT: **Effects of testosterone and 17-beta-estradiol on TNF-alpha-induced E-selectin and**

- VCAM-1 expression in endothelial cells. Analysis of the underlying receptor pathways.** *Life Sci* 2002, **71**: 15-29.
21. Caulin-Glaser T, Watson CA, Pardi R, Bender JR: **Effects of 17beta-estradiol on cytokine-induced endothelial cell adhesion molecule expression.** *J Clin Invest* 1996, **98**: 36-42.
 22. Mori M, Tsukahara F, Yoshioka T, Irie K, Ohta H: **Suppression by 17beta-estradiol of monocyte adhesion to vascular endothelial cells is mediated by estrogen receptors.** *Life Sci* 2004, **75**: 599-609.
 23. Simoncini T, Maffei S, Basta G, Barsacchi G, Genazzani AR, Liao JK, De Caterina R: **Estrogens and glucocorticoids inhibit endothelial vascular cell adhesion molecule-1 expression by different transcriptional mechanisms.** *Circ Res* 2000, **87**: 19-25.
 24. Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, Shine J: **Sequence and expression of human estrogen receptor complementary DNA.** *Science* 1986, **231**: 1150-1154.
 25. Kuiper GG, Enmark E, Peltö-Huikko M, Nilsson S, Gustafsson JA: **Cloning of a novel receptor expressed in rat prostate and ovary.** *Proc Natl Acad Sci U S A* 1996, **93**: 5925-5930.
 26. Walter P, Green S, Greene G, Krust A, Bornert JM, Jeltsch JM, Staub A, Jensen E, Scrace G, Waterfield M, .: **Cloning of the human estrogen receptor cDNA.** *Proc Natl Acad Sci U S A* 1985, **82**: 7889-7893.
 27. Lindner V, Kim SK, Karas RH, Kuiper GG, Gustafsson JA, Mendelsohn ME: **Increased expression of estrogen receptor-beta mRNA in male blood vessels after vascular injury.** *Circ Res* 1998, **83**: 224-229.
 28. Evans MJ, Harris HA, Miller CP, Karathanasis SK, Adelman SJ: **Estrogen receptors alpha and beta have similar activities in multiple endothelial cell pathways.** *Endocrinology* 2002, **143**: 3785-3795.
 29. Tyree CM, Zou A, Allegretto EA: **17beta-Estradiol inhibits cytokine induction of the human E-selectin promoter.** *J Steroid Biochem Mol Biol* 2002, **80**: 291-297.
 30. Nakamura Y, Suzuki T, Miki Y, Tazawa C, Senzaki K, Moriya T, Saito H, Ishibashi T, Takahashi S, Yamada S, Sasano H: **Estrogen receptors in atherosclerotic human aorta: inhibition of human vascular smooth muscle cell proliferation by estrogens.** *Mol Cell Endocrinol* 2004, **219**: 17-26.
 31. Wilson ME, Rosewell KL, Kashon ML, Shughrue PJ, Merchenthaler I, Wise PM: **Age differentially influences estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) gene expression in specific regions of the rat brain.** *Mech Ageing Dev* 2002, **123**: 593-601.
 32. Losordo DW, Kearney M, Kim EA, Jekanowski J, Isner JM: **Variable expression of the estrogen receptor in normal and atherosclerotic coronary arteries of premenopausal women.** *Circulation* 1994, **89**: 1501-1510.
 33. Adams MR, Register TC, Golden DL, Wagner JD, Williams JK: **Medroxyprogesterone acetate antagonizes inhibitory effects of conjugated equine estrogens on coronary artery atherosclerosis.** *Arterioscler Thromb Vasc Biol* 1997, **17**: 217-221.
 34. Clarkson TB, Anthony MS, Jerome CP: **Lack of effect of raloxifene on coronary artery atherosclerosis of postmenopausal monkeys.** *J Clin Endocrinol Metab* 1998, **83**: 721-726.

35. Clarkson TB, Anthony MS, Morgan TM: **Inhibition of postmenopausal atherosclerosis progression: a comparison of the effects of conjugated equine estrogens and soy phytoestrogens.** *J Clin Endocrinol Metab* 2001, **86**: 41-47.
36. Williams JK, Anthony MS, Honore EK, Herrington DM, Morgan TM, Register TC, Clarkson TB: **Regression of atherosclerosis in female monkeys.** *Arterioscler Thromb Vasc Biol* 1995, **15**: 827-836.
37. Mautino MR, Ramsey WJ, Reiser J, Morgan RA: **Modified human immunodeficiency virus-based lentiviral vectors display decreased sensitivity to trans-dominant Rev.** *Hum Gene Ther* 2000, **11**: 895-908.
38. Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L: **Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences.** *Nat Genet* 2000, **25**: 217-222.
39. Sirven A, Pflumio F, Zennou V, Titeux M, Vainchenker W, Coulombel L, Dubart-Kupperschmitt A, Charneau P: **The human immunodeficiency virus type-1 central DNA flap is a crucial determinant for lentiviral vector nuclear import and gene transduction of human hematopoietic stem cells.** *Blood* 2000, **96**: 4103-4110.
40. Seppen J, Rijnberg M, Cooreman MP, Oude Elferink RP: **Lentiviral vectors for efficient transduction of isolated primary quiescent hepatocytes.** *J Hepatol* 2002, **36**: 459-465.
41. Seppen J, Barry SC, Klinkspoor JH, Katen LJ, Lee SP, Garcia JV, Osborne WR: **Apical gene transfer into quiescent human and canine polarized intestinal epithelial cells by lentivirus vectors.** *J Virol* 2000, **74**: 7642-7645.
42. Back NK, Nijhuis M, Keulen W, Boucher CA, Oude Essink BO, van Kuilenburg AB, van Gennip AH, Berkhout B: **Reduced replication of 3TC-resistant HIV-1 variants in primary cells due to a processivity defect of the reverse transcriptase enzyme.** *EMBO J* 1996, **15**: 4040-4049.
43. Hoekstra M, Kruijt JK, Van Eck M, van Berkel TJ: **Specific gene expression of ATP-binding cassette transporters and nuclear hormone receptors in rat liver parenchymal, endothelial, and Kupffer cells.** *J Biol Chem* 2003, **278**: 25448-25453.

