

Estrogen Receptor–Mediated, Nitric Oxide–Dependent Modulation of the Immunologic Barrier Function of the Endothelium

Regulation of Fas Ligand Expression by Estradiol

Carole Amant, PhD; Pernille Holm, MD; Shu-hua Xu, MS; Neil Tritman, BA; Marianne Kearney, BS; Douglas W. Losordo, MD

Background—Premenopausal women have a lower incidence of coronary artery disease than postmenopausal women or same-age men. Although the mechanisms of this apparent relative protection against atherosclerosis remain ill defined, estradiol, which is present in higher concentrations before menopause, is considered to play a central role. Recently, Fas ligand (FasL) expression by the vascular endothelium has been shown to inhibit the migration of inflammatory cells into the vessel wall, an event that is considered crucial for the development of atherosclerosis.

Methods and Results—The regulation of endothelial FasL expression by estradiol was investigated in vivo and in vitro. In an ovariectomized, cholesterol-clamped rabbit model, FasL expression was shown to be downregulated by elevations in serum cholesterol, which also resulted in invasion of the arterial wall by macrophages. Estradiol replacement resulted in restoration of FasL expression, with resultant inhibition of leukocyte traffic across the endothelium. Inhibition of NO production by addition of L-NAME to the drinking water of the estradiol-treated rabbits abrogated these effects. In vitro, estradiol is shown to regulate FasL expression at the transcriptional level via an estrogen receptor–mediated, NO-dependent mechanism.

Conclusions—Estradiol transcriptionally regulates endothelial FasL expression by a mechanism involving at least one of the estrogen receptors. In an animal model of atherosclerosis, estradiol restores FasL expression, which is suppressed by atherogenic levels of serum cholesterol. The maintenance of endothelial FasL expression by estradiol may represent a mechanism of estrogen's apparent antiatherogenic effect. (*Circulation*. 2001;104:2576-2581.)

Key Words: atherosclerosis ■ endothelium ■ cells ■ coronary disease

Menopause is marked by a sudden decrease in serum 17β -estradiol (E2) levels and by an increase in the incidence of coronary artery disease. A large volume of epidemiological and observational data in humans¹ as well as in vitro² and in vivo³⁻⁷ experimentation has suggested that the decrease in E2 production is not a coincidence but rather a causal factor in atherogenesis in postmenopausal women. Despite this intense scrutiny, however, whether and how estrogen inhibits atherosclerosis remains as unclear as the genesis of the disease itself.¹

Inflammatory cells are an established component of atheromatous lesions. Attention has focused on these cells as mediators, if not initiators, of atherosclerosis, with inflammatory cell invasion of the vessel wall considered a potentially seminal event in atherogenesis. Inhibition of leukocyte traffic across the endothelium, therefore, might be considered atheroprotective. Indeed, recent data suggest that estrogen is

capable of inhibiting leukocyte extravasation⁸; however, the mechanism of this effect has not been completely defined.

Fas ligand (FasL) is a membrane protein capable of inducing apoptosis in cells bearing its receptor, Fas, primarily cells of the immune system. It was recently shown that FasL is expressed by arterial endothelial cells (ECs) and that tumor necrosis factor- α downregulates FasL expression associated with an increase in inflammatory cell migration into the vessel wall. Constitutive expression of FasL in these ECs, achieved with an adenoviral vector, inhibited leukocyte traffic into the arterial wall, apparently by inducing apoptosis of Fas-bearing inflammatory cells.⁹ In addition, FasL-deficient mice have been shown to display enhanced leukocyte infiltration into the vessel wall.¹⁰ These studies suggested a key role for FasL in maintaining an active barrier function of the endothelium against leukocyte invasion.

Studies investigating the role of E2 as a potential atheroprotective agent have shown it to inhibit both atherogenesis

Received June 28, 2001; revision received September 5, 2001; accepted September 6, 2001.

From the Divisions of Cardiovascular Medicine and Cardiovascular Research, St Elizabeth's Medical Center, Boston, Mass, and Novo Nordisk (P.H.), Malov, Denmark.

Correspondence to Douglas W. Losordo, MD, Divisions of Cardiovascular Medicine and Cardiovascular Research, St Elizabeth's Medical Center, 736 Cambridge St, Boston, MA 02135. E-mail dlosordo@opal.tufts.edu

© 2001 American Heart Association, Inc.

Circulation is available at <http://www.circulationaha.org>

and leukocyte extravasation in animal models of atherosclerosis. Accordingly, we performed a series of experiments to test the hypothesis that E2 regulates endothelial FasL expression as a potential mechanism for the inhibition of atherogenesis in premenopausal women.

Methods

In Vivo Experimental Design

In vivo studies were performed on 64 “cholesterol-clamped” ovariectomized female New Zealand White rabbits. The cholesterol-clamped rabbit model yields equivalent serum cholesterol levels by dietary manipulation of each rabbit, thereby nullifying the cholesterol-lowering effect of E2 in treated subgroups,¹¹ permitting investigation of the nonlipid (ie, “direct”) effects of E2 on vascular biology. The animals were randomly assigned in equal numbers to 1 of 4 treatment groups: (1) control, (2) E2 given subcutaneously in a concentration of 50 $\mu\text{g}/\text{kg}$ every third day, (3) E2 plus NG-nitro-L-arginine methyl ester (L-NAME) 160 mg/mL added to the drinking water (previously shown to attenuate NO production),¹² and (4) L-NAME alone. This dosing regimen of E2 achieves plasma levels of 467 ± 26 to 601 ± 26 pmol/L, which are equivalent to 106 to 163 pg/mL, well within the human physiological range.^{11,13} After 4 weeks, the aortas were harvested with part of the thoracic aorta frozen for Western analysis and the remainder fixed in methanol for immunostaining. For comparison, the aortas of intact rabbits fed a normal diet were also harvested. Matched sections of the upper thoracic aorta were submitted for immunostaining.

Cell Culture

Human umbilical vein ECs (HUVECs) were isolated as previously described,² plated onto 1.5% gelatin-coated wells, and grown in phenol red-free medium 199 (M199) with 20% FBS with a defined E2 content of 7.6 pg/mL or 2.8×10^{-11} mol/L and bovine brain extracts. For the cytotoxicity assays, HUVECs were incubated in the presence or absence of 10 $\mu\text{g}/\text{mL}$ of matrix metalloproteinase *p*-aminobenzoyl-Gly-Pro-D-Leu-D-Ala hydroxamic acid (Sigma Chemical Co). The matrix metalloproteinase inhibitor was added to ensure that cytotoxicity was the result of membrane-bound, rather than soluble, FasL.

Antibodies

FasL antibody (Q-20) was purchased from Santa Cruz Biotechnology, and anti-human FasL mouse monoclonal antibody (clone 33) was purchased from Transduction Laboratories. Monoclonal antibody against human CD31 (platelet and endothelial cell adhesion molecule, PECAM-1) and mouse Ram-11 antibody were purchased from Dako and human anti- α -tubulin from Santa Cruz Biotechnology. Anti-Fas FITC was obtained from Pharmingen. Monoclonal antibody against human CD31 (PECAM-1) and mouse Ram-11 antibody were purchased from Dako and human anti- α -tubulin from Santa Cruz Biotechnology. Anti-Fas FITC was obtained from Pharmingen.

Immunohistochemistry

After fixation in 100% methanol, sections from matching regions of the upper thoracic aorta from all animals were stained for the presence of macrophages by use of Ram-11 antibody. Ten randomly chosen sections from each treatment group were then evaluated by a blinded observer, and the numbers of infiltrating macrophages were counted.

Quantitative RT-PCR

Total RNA was isolated from HUVECs and from Jurkat cells (quiescent and activated with phorbol 12-myristate 13-acetate [PMA]/ionomycin)¹⁴ with Totally RNA according to the manufacturer’s directions (Ambion). All of the components for the reverse transcription (RT) procedure were purchased from Gibco-BRL Life Technologies. We used 25 pmol each of 3’ and 5’ FasL intron-spanning primers: sense

primer 5’-AAGAAGAGAGGGAACCCACAGCAC-3’ and reverse primer 5’-CCGAAAAACGTCTGAGATTCCTC-3’ yielding a 617-bp reaction product. To quantify the FasL mRNA product, we used the “competimer” quantitative polymerase chain reaction (PCR) technique: FasL cDNA and 18S cDNA were coamplified at the same time for each sample. PCR products were separated on agarose gels containing ethidium bromide and quantified with integrated density analysis software (EagleSight Software 3.2, Stratagene). RT-PCR and relative quantification of PCR products were performed ≥ 3 times on each sample of RNA.

Western Blotting

Western analysis of tissue homogenates of rabbit aorta was performed as previously described.¹⁵

Transfection

Transient transfection assays with a luciferase reporter containing a 2365-bp segment of the FasL 5’ flanking sequence were performed to evaluate regulation of FasL promoter activity and were performed as previously described.¹⁵ FasL reporter plasmid was the generous gift of C. Paya (Mayo Clinic, Rochester, Minn), and estrogen receptor (ER)- α expression plasmid was the generous gift of Pierre Chambon (INSERM, Illkirch France). Transfections were performed in triplicate and repeated twice.

Cytotoxicity Assay

To verify that the FasL expressed by ECs under the direction of E2 was functional, cytotoxicity assays were performed. HUVECs were plated in 96-well plates at a density of 5×10^4 cells per dish and were allowed to attach overnight. Cells were washed twice with PBS and coincubated with 10^5 Jurkat cells in M199 plus 2% defined ($< 10^{-10}$ mol/L E2) FBS in the presence of one of the following conditions; E2 10^{-8} mol/L alone; E2 10^{-8} mol/L plus recombinant Fas:Fc 2 $\mu\text{g}/\text{mL}$ in the presence of the enhancer 1 $\mu\text{g}/\text{mL}$ (Alexis Corp); and E2 10^{-8} mol/L plus L-NAME 10^{-5} mol/L. Supernatants were harvested after 24 hours.

Cytospin cell preparations of the Jurkat cells were fixed in 4% paraformaldehyde at room temperature for 1 hour to perform terminal dUTP nick end-labeling (TUNEL) assays according to the manufacturer’s specifications (Roche). Slides were rinsed 3 times with PBS, and samples were directly analyzed under a fluorescence microscope. The number of positively labeled nuclei in 3 randomly selected microscopic fields were counted and divided by the total cell count to determine the percentage of apoptotic cells. The cytotoxicity assay was repeated 3 times.

Flow Cytometry

To determine whether E2 treatment (in the above cytotoxicity assay) resulted in changes in the cell-surface expression of Fas in Jurkat cells, a portion of the cells were centrifuged and incubated 30 minutes at 4°C with PBS, 10% FBS. They were then incubated with specific Fas antibody diluted with 1% BSA and 0.1% NaN_3 in PBS at 4°C for 60 minutes. Isotype-matched nonspecific immunoglobulins were used as negative controls. Immunofluorescence staining was analyzed by a fluorescence-activated cell sorter (FACS; EPICS/XL).

Statistics

All results are reported as mean \pm SEM. Statistical comparisons between groups were made by ANOVA, which was followed by a 2-tailed unpaired Student’s *t* test for comparisons between the means of 2 groups. A value of $P < 0.05$ was interpreted to denote statistical significance.

Results

E2 Restores FasL Expression in Aortic Endothelium in Hypercholesterolemic Rabbit

Under control conditions with a normal diet, FasL is abundantly expressed in the aortic endothelium (Figures 1 and 2).

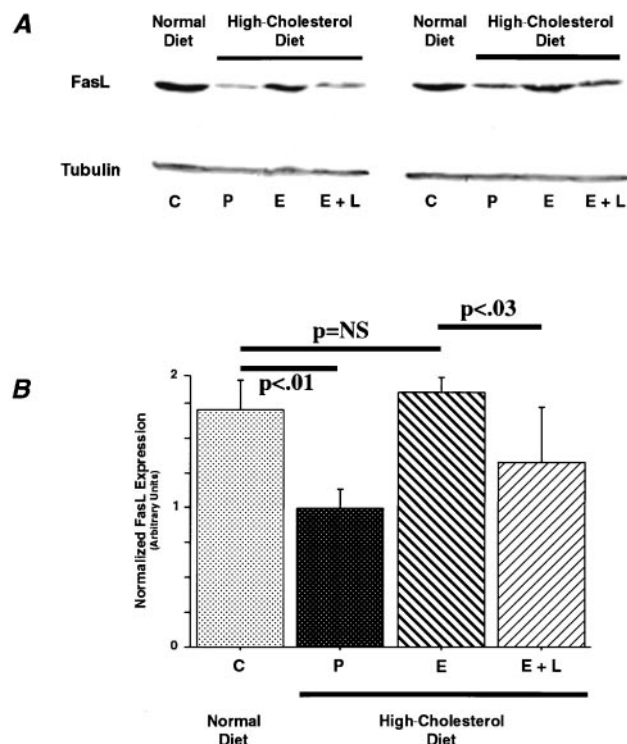


Figure 1. E2 regulates FasL protein expression in rabbit aorta by an NO-dependent pathway. A, Representative Western blots of protein extracts from rabbit aorta. Under control conditions with normal diet (C), FasL is abundantly expressed. Cholesterol feeding results in marked reduction of FasL expression in placebo (P) group that is restored by E2 (E) treatment. Recovery of FasL expression is blocked in E2-treated animals whose drinking water was supplemented with L-NAME (E+L). B, Quantification of Western analysis. Western blots for FasL and tubulin were scanned into a computerized analysis system. Normalization of FasL expression to tubulin expression for each individual sample therefore permitted quantitative comparison of FasL expression under different conditions. FasL expression is significantly reduced by hypercholesterolemia ($P < 0.01$). E2 treatment results in restoration of FasL expression ($P < 0.01$ vs placebo) to levels similar to baseline, normal diet ($P = \text{NS}$ vs normal diet), although cholesterol level in E2-treated animals was maintained at high levels by dietary intervention. L-NAME attenuates restoration of FasL expression by E2 ($P < 0.03$ vs E2 alone).

After 4 weeks of cholesterol feeding, FasL expression is decreased in ovariectomized rabbits (Figure 1A and 1B). Thus, hypercholesterolemia, a risk factor for human atherosclerosis and the substrate for atheroma formation in this animal model, results in a decrease in FasL expression by the aortic endothelium under conditions of estrogen depletion. Replacement treatment with E2, which inhibits atherogenesis in this model, results in restoration of FasL expression (Figure 1A and 1B). It is important to note that the restoration of FasL expression occurs despite persistent elevations of serum cholesterol, achieved by individual dietary manipulation in the cholesterol-clamped model.¹¹ In contrast, FasL expression is not restored when estrogen replacement is accompanied by inhibition of NO production by the NO synthase competitor L-NAME (Figure 1A and 1B). Quantification of these results normalized to tubulin expression is shown in Figure 1B. The localization of FasL expression in the aortic endothelium was verified by immunohistochemistry (Figure 2).

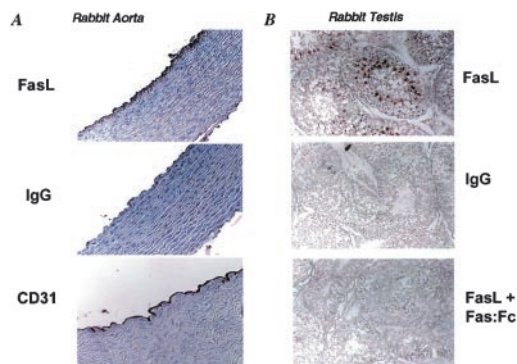


Figure 2. FasL expression in aorta is localized to ECs. Immunostaining of sections of rabbit aorta (A) reveals expression of FasL restricted to EC layer (top). Adjacent cryosection was stained for CD31 (bottom), an antigen expressed by ECs. Rabbit testis (B) is included as positive control showing specific FasL staining of Sertoli cells. Specificity of FasL staining was demonstrated by substituting nonspecific goat IgG at same concentration (middle) or by preincubation of sections with a recombinant Fas protein (Fas:Fc) (B, bottom).

E2-Mediated Increase in FasL Expression Is Associated With Inhibition of Transendothelial Leukocyte Traffic

To determine whether the alterations in FasL expression were accompanied by changes in inflammatory cell infiltration of the aorta as previously shown,^{9,10} sections of each aorta were stained with the rabbit macrophage marker Ram 11 (Figure 3A). The total number of adherent and infiltrating Ram 11-positive cells in 10 randomly selected sections were counted by a blinded observer. These results, consistent with previous reports,⁸ reveal that E2 inhibits inflammatory cell migration into the aorta and that this inhibitory effect is blocked when NO production is blocked by L-NAME (Figure 3B) (mean number of macrophages per artery): placebo, 4.86 ± 1.7 versus E2, 0.167 ± 0.115 ($P < 0.03$ versus placebo)

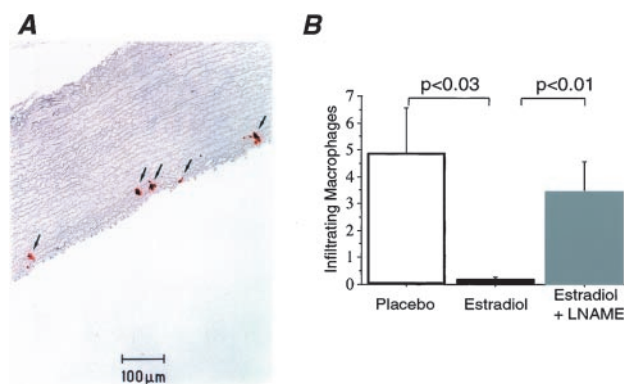


Figure 3. Restoration of aortic endothelial FasL expression inhibits leukocyte extravasation. Sections of aorta from all animals were stained with rabbit macrophage marker Ram 11. A, Representative example of Ram 11-stained section of rabbit aorta from placebo-treated animal reveals evidence of macrophages in media. B, Quantification of adherent and infiltrating cells reveals hypercholesterolemia-induced macrophage infiltration that is attenuated by E2 treatment ($P < 0.03$ vs placebo). Protective effect of E2 treatment was inhibited by L-NAME ($P < 0.01$ vs E2 alone), consistent with changes in endothelial FasL expression induced by E2 and L-NAME, respectively.

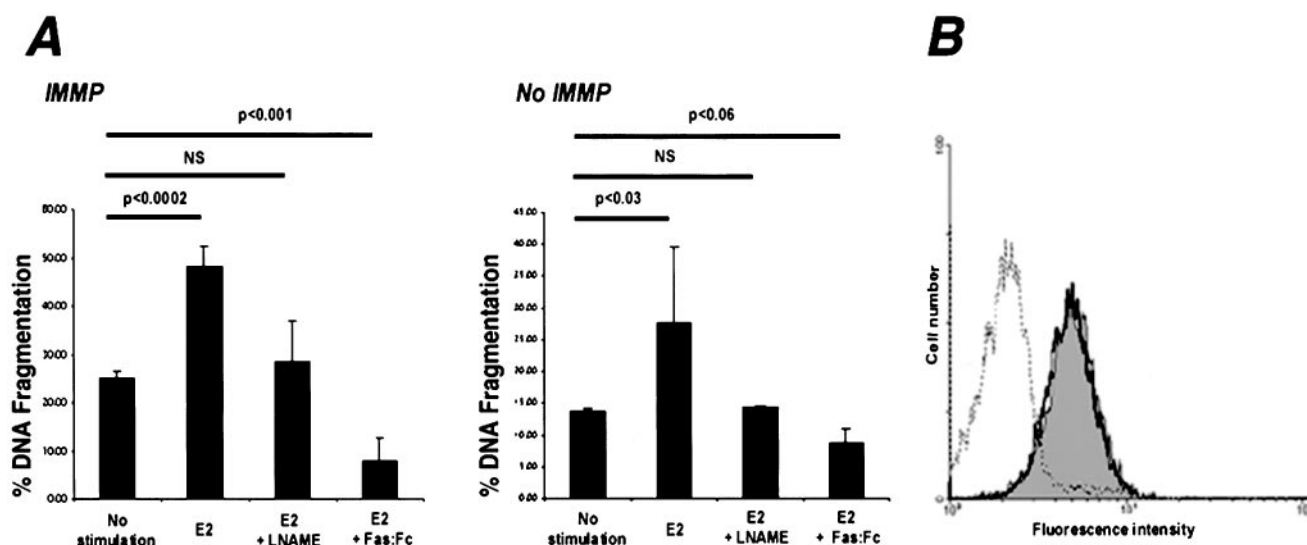


Figure 4. Cytotoxicity assay verifies expression of functional FasL induced by E2. A, Coculture of Fas-expressing Jurkat cells with ECs was performed to verify that FasL expressed by ECs under direction of E2 was capable of appropriately inducing apoptosis in cells bearing Fas receptor. TUNEL assays performed on Jurkat cells cocultured with ECs for 20 hours in presence (left) or absence (right) of inhibitor of metalloproteinase (IMMP) reveal a significant increase in apoptosis of Fas-bearing Jurkat cells after exposure of ECs to E2. E2-induced increase in FasL-mediated apoptosis is inhibited by L-NAME, consistent with a reduction in EC FasL expression when NO production is blocked. To verify that increase in apoptosis was FasL-mediated, Fas:Fc was added with E2 and inhibited induction of apoptosis. Jurkat cells incubated with apoptosis-inducing antibody CH11 were included as positive controls (data not shown). Cytotoxicity assays on HUVECs alone were also performed as negative controls (data not shown). B, Flow cytometry reveals no change in Fas expression on surface of Jurkat cells incubated in absence (solid curve) or presence (dotted curve) of E2. Increase in EC apoptosis shown in coculture assay is therefore not result of E2-mediated increase in Jurkat cell Fas expression and can only be due to demonstrated increase in EC FasL expression. FACS with control antibody (mouse IgM FITC) is shown in shaded curve.

versus E2 plus L-NAME, 3.42 ± 1.1 ($P < 0.01$ versus E2 alone, $P = \text{NS}$ versus placebo). The changes in macrophage infiltration, therefore, paralleled E2-mediated changes in endothelial FasL expression, consistent with the previously documented function of endothelial FasL as an active barrier to inflammatory cell migration.⁹

E2 Induces Functional EC FasL Expression

We next sought to determine whether the FasL expressed by ECs under the direction of E2 was functional and therefore capable of inducing apoptosis of adherent inflammatory cells. We used Jurkat cells, which constitutively express the Fas receptor, in a coculture assay with HUVECs in the presence or absence of E2 to determine whether the increase in EC FasL expression induced by E2 resulted in increased apoptosis of Fas-bearing cells (Figure 4).

FasL is expressed by HUVECs under basal conditions,⁹ so coculture with Jurkat cells results in a low level of Jurkat apoptosis at baseline (Figure 4A). In the presence of E2, Jurkat apoptosis increases 2-fold. This increase in apoptosis is completely blocked by the addition of L-NAME to the culture medium, consistent with the effect of NOS inhibition of FasL expression in vivo (Figure 1) and consistent with the trend observed in vitro (Figure 5). To further verify that the increase in apoptosis induced by E2 was Fas-FasL-mediated, a parallel series of coculture experiments was performed with the addition of Fas:Fc (which inhibits Fas-FasL-induced apoptosis). These studies reveal complete attenuation of the E2-induced increase in Jurkat apoptosis (Figure 4A). Finally, to exclude an increase in Jurkat Fas expression as the cause of the observed increase in apoptosis, FACS analysis of identi-

cally treated cells was performed and revealed no change in Jurkat cell Fas expression in the presence of E2 (Figure 4B). E2 also did not induce Jurkat apoptosis in the absence of HUVECs (data not shown).

Transcriptional Regulation of FasL by E2

We next investigated the regulation of FasL mRNA expression by E2 using semiquantitative RT-PCR (Figure 5). First, RT-PCR was performed on Jurkat cells stimulated with PMA/ionomycin (used as positive control for FasL mRNA expression), unstimulated Jurkat cells (used as a negative control), and HUVECs, verifying primer specificity and that HUVECs express FasL mRNA as previously observed⁹ (Figure 5A). Next, quantitative-competitive RT-PCR was performed. Coamplification of FasL and 18S mRNA resulted in 2 distinct bands (Figure 5B). Densitometry of FasL RT-PCR product/18S RT-PCR product reveals a nearly 3-fold induction of FasL mRNA levels by E2 compared with control ($P < 0.0003$, Figure 5C, lanes 3 and 2, respectively). L-NAME (lane 4) does not alter the baseline expression of FasL but attenuates E2-induced FasL mRNA upregulation (lane 5) (L-NAME plus E2 versus E2, $P < 0.0005$). These data, suggesting that E2 regulates EC FasL expression via an NO-mediated mechanism, are consistent with the regulation of aortic EC FasL expression shown in vivo above (Figure 1) and with the cytotoxicity assays (Figure 4).

To determine whether the regulation of FasL mRNA expression by E2 occurred at the transcriptional level, we next performed a series of transient transfection assays using a luciferase reporter construct containing the FasL 5' flanking sequence cotransfected with ER- α . E2 had no effect on FasL

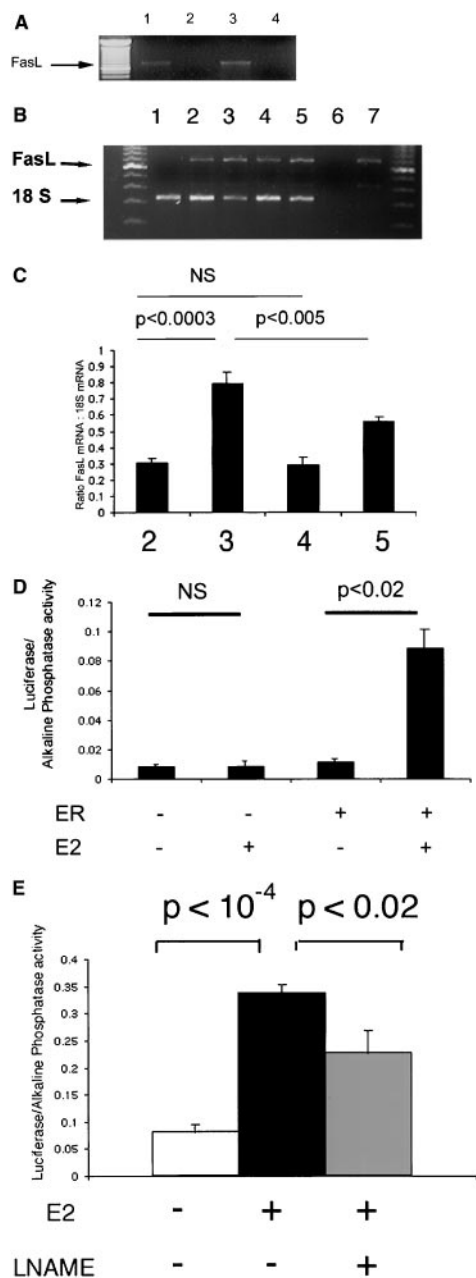


Figure 5. FasL expression is transcriptionally regulated by E2. A, RT-PCR on RNA from PMA/ionomycin-activated Jurkat cells (lane 1), nonactivated Jurkat cells (lane 2), HUVECs (lane 3), and RT-PCR negative control (lane 4) was performed first to verify primer specificity and expression of FasL in HUVECs. There is no FasL expression in Jurkat cells in absence of stimulation with PMA/ionomycin. B, Competitive RT-PCR reveals increase in FasL mRNA expression by E2. Representative gel showing coamplification of FasL mRNA (617-bp product) and 18S mRNA (488-bp product). Quantitative-competitive RT-PCR reveals that increased FasL expression in HUVECs treated with E2 (lane 3) vs control (lane 2) L-NAME alone does not effect basal FasL expression (lane 4) but attenuates induction of FasL by E2 (lane 5). Controls included amplification of 18S alone (lane 1), no DNA (lane 6), and FasL primers alone (lane 7). C, Quantification by computerized densitometric analysis reveals a significant increase in FasL mRNA expression by ECs induced by E2 ($P < 0.0003$ vs control) that is inhibited by L-NAME ($P < 0.005$ vs E2 alone). Ratio of FasL mRNA/18S mRNA density values was obtained by scanning densitometer and represents mean of 3 separate experiments. D, FasL transcriptional regulation by

promoter activity in the absence of ER (Figure 5D). In the presence of ER α , E2 caused a significant increase in FasL promoter activity, which was attenuated in the presence of L-NAME (Figure 5E), paralleling the changes seen in mRNA and protein expression as well as the functional assay.

Discussion

These data suggest a novel mechanism for the inhibition of atherosclerosis by E2: preservation of the integrity of an active barrier function of the endothelium by maintenance of endothelial FasL expression. The potential role of FasL in preventing leukocyte extravasation has been shown previously.⁹ Our data reveal that hypercholesterolemia, a risk factor for atherosclerosis and a precondition for atheroma formation in the rabbit model, reduces FasL expression in vivo, with an associated increase in macrophage infiltration of the arterial wall. E2, assumed to be the atheroprotective factor in premenopausal women, restores/maintains FasL expression and thereby inhibits leukocyte traffic across the endothelium despite the ongoing presence of severely elevated serum cholesterol levels.

In vivo, 2 forms of FasL can be found: a membrane-bound (42 to 48 kDa) and a soluble (sFasL, 26 kDa) FasL form resulting from the release of the extracellular domain of membrane-bound FasL cleaved by metalloproteinases.¹⁶ The membrane and soluble forms of human FasL exhibit differential cytotoxic activities. The ability of FasL to induce apoptosis is mediated primarily by the membrane form of the protein.¹⁷ FasL works locally via cell-cell interactions under physiological conditions, and the purpose of shedding FasL to its soluble form appears to be to attenuate cytotoxicity.¹⁸ The results of the cytotoxicity assay reported here suggest that E2 increased endothelial expression of the active FasL, ie, expressed on the cell membrane.

The regulation of endothelial FasL expression by E2 occurs, at least in part, at the transcriptional level, mediated by direct effects on the FasL promoter via ER- α . The FasL promoter contains both estrogen-responsive elements, induced via ER- α in the presence of E2, and AP-1 sites, which have been shown to be regulated by the more recently discovered ER- β .¹⁹ Because both of these receptors have been reported to be expressed by ECs, the possibility for complex regulatory control by various naturally occurring and synthetic estrogens and estrogen-like compounds is evident. The possibility of other mechanisms regulating FasL expression in vivo is also suggested by the difference between the regulation of FasL

estrogen is ER-dependent. HeLa cells were transiently transfected with a FasL promoter reporter plasmid \pm ER- α expression plasmid and incubated with medium alone or medium + E2 10^{-8} mol/L. E2 induces significant FasL promoter activity only in presence of cotransfected ER- α . E, FasL transcriptional regulation by estrogen is mediated by NO. Cos-1 cells were transiently transfected with a FasL promoter reporter plasmid plus ER- α expression plasmid and incubated with medium alone, medium plus E2 10^{-8} mol/L, or medium + E2 10^{-8} mol/L + L-NAME 10^{-5} mol/L. In presence of ER- α , E2 induces significant FasL promoter activity ($P < 10^{-4}$ vs control) that is inhibited when NO production is blocked by L-NAME ($P < 0.02$ vs E2 alone).

mRNA expression in vitro and the phenotypic differences demonstrated in vivo.

These data also show that NO plays a central role in at least one E2-mediated signaling pathway of FasL expression. The ability of the endothelium to release NO in response to certain stimuli^{20–24} has been equated with normal endothelial function but has previously been considered important largely for its vasodilator function. The fact that NO appears to be essential for E2-mediated regulation of FasL expression and maintenance of the immunologic barrier function of the endothelium implicates an alternative model. Our findings suggest that NO is important as a signaling molecule, in this case integral in maintaining FasL-mediated inhibition of leukocyte invasion of the underlying arterial wall. This association is consistent with the recent finding that atherosclerosis-prone apolipoprotein E $-/-$ mice with targeted disruption of eNOS expression develop more severe atherosclerotic lesions.²⁵ In addition, the recent description of a membrane-bound estrogen receptor capable of inducing NO release via an AKT-dependent pathway^{26,27} raises interesting possibilities regarding multiple mechanisms of E2-dependent regulation of FasL expression.

Nathan et al²⁸ previously showed that decreases in MCP-1 expression induced by E2 were associated with decreased inflammatory cell recruitment to the subendothelium. These findings suggested that E2 acted by impairing attraction and adherence of circulating leukocytes. The present results suggest the possibility of a more active role for E2 in maintaining arterial integrity, preserving the expression of a surface molecule capable of inducing apoptosis of adherent inflammatory cells.

Randomized clinical trials have failed to verify the coronary protective effect of certain forms of hormone replacement therapy^{29,30} that was strongly suggested by observational studies. This discrepancy underscores the need for a more complete understanding of the mechanisms that result in the lower incidence of coronary events in premenopausal women. The present findings identify a novel pathway for the inhibition of nascent atherosclerosis by E2 and also suggest another mechanism by which E2-mediated release of NO may contribute to the normal functioning of the endothelium.

Acknowledgments

This work was funded in part by NIH grants HL-63414, HL-63695, and AG-16332 (Dr Losordo) and by grants from the Association pour la Recherche sur le Cancer and the Institut National de la Santé et de la Recherche Médicale (Dr Amant).

References

- Mendelsohn ME, Karas RH. The protective effects of estrogen on the cardiovascular system. *N Engl J Med*. 1999;340:1801–1811.
- Spyridopoulos I, Sullivan AB, Kearney M, et al. Estrogen receptor-mediated inhibition of human endothelial cell apoptosis: estradiol as a survival factor. *Circulation*. 1997;95:1505–1514.
- Adams MR, Kaplan JR, Manuck SB, et al. Inhibition of coronary artery atherosclerosis by 17-beta estradiol in ovariectomized monkeys: lack of an effect of added progesterone. *Arteriosclerosis*. 1990;10:1051–1057.
- Foegh ML, Asotra S, Howell MH, et al. Estradiol inhibition of arterial neointimal hyperplasia after balloon injury. *J Vasc Surg*. 1994;19:722–726.
- Sullivan TR, Karas RH, Aronovitz M, et al. Estrogen inhibits the response-to-injury in a mouse carotid artery model. *J Clin Invest*. 1995;95:2482–2488.
- Krasinski K, Spyridopoulos I, Asahara T, et al. Estradiol accelerates functional endothelial recovery after arterial injury. *Circulation*. 1997;95:1768–1772.
- Iafrafi MD, Karas RH, Aronovitz M, et al. Estrogen inhibits the vascular injury response in estrogen receptor-deficient mice. *Nat Med*. 1997;3:545–548.
- Nathan L, Pervin S, Singh R, et al. Estradiol inhibits leukocyte adhesion and transendothelial migration in rabbits in vivo: possible mechanisms for gender differences in atherosclerosis. *Circ Res*. 1999;85:377–385.
- Sata M, Walsh K. TNF α regulation of Fas ligand expression on the vascular endothelium modulates leukocyte extravasation. *Nat Med*. 1998;4:415–420.
- Sata M, Walsh K. Fas ligand-deficient mice display enhanced leukocyte infiltration and intima hyperplasia in flow-restricted vessels. *J Mol Cell Cardiol*. 2000;32:1395–1400.
- Holm P, Stender S, Andersen HO, et al. Antiatherogenic effect of estrogen abolished by balloon catheter injury in cholesterol-clamped rabbits. *Arterioscler Thromb Vasc Biol*. 1997;17:1504–1511.
- Holm P, Andersen HL, Andersen MR, et al. The direct antiatherogenic effect of estrogen is present, absent, or reversed, depending on the state of the arterial endothelium: a time course study in cholesterol-clamped rabbits. *Circulation*. 1999;100:1727–1733.
- Holm P, Andersen HO, Nordestgaard BG, et al. Effect of estrogen replacement therapy on development of experimental arteriosclerosis: a study in transplanted and balloon-injured rabbit aortas. *Atherosclerosis*. 1995;115:191–200.
- Faris M, Kokot N, Latinis K, et al. The c-Jun N-terminal kinase cascade plays a role in stress-induced apoptosis in Jurkat cells by up-regulating Fas ligand expression. *J Immunol*. 1998;160:134–144.
- Spyridopoulos I, Principe N, Krasinski KL, et al. Restoration of E2F expression rescues vascular endothelial cells from tumor necrosis factor- α -induced apoptosis. *Circulation*. 1998;98:2883–2890.
- Tanaka M, Suda T, Haze K, et al. Fas ligand in human serum. *Nat Med*. 1996;2:317–322.
- Suda T, Hashimoto H, Tanaka M, et al. Membrane Fas ligand kills human peripheral blood T lymphocytes, and soluble Fas ligand blocks the killing. *J Exp Med*. 1997;186:2045–2050.
- Nagata S. Apoptosis by death factor. *Cell*. 1997;88:355–365.
- Paeck K, Webb P, Kuiper GGJM, et al. Differential ligand activation of estrogen receptors ER α and ER β at AP1 sites. *Science*. 1997;277:1508–1510.
- Lantin-Hermoso RL, Rosenfeld CR, Yuhanna IS, et al. Estrogen acutely stimulates nitric oxide synthase activity in fetal pulmonary artery endothelium. *Am J Physiol*. 1997;273:L119–L126.
- Sudhir K, Chou TM, Messina LM, et al. Endothelial dysfunction in a man with disruptive mutation in oestrogen-receptor gene. *Lancet*. 1997;349:1146–1147.
- Wang XF, Barber DA, Lewis DA, et al. Gender and transcriptional regulation of NO synthase and ET-1 in porcine aortic endothelial cells. *Am J Physiol*. 1997;273:H1962–H1967.
- Chen Z, Yuhanna IS, Galcheva-Gargova Z, et al. Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J Clin Invest*. 1999;103:401–406.
- Russell KS, Haynes MP, Sinha D, et al. Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. *Proc Natl Acad Sci U S A*. 2000;97:5930–5935.
- Knowles JW, Reddick RL, Jennette JC, et al. Enhanced atherosclerosis and kidney dysfunction in eNOS(-/-)Apoe(-/-) mice are ameliorated by enalapril treatment. *J Clin Invest*. 2000;105:451–458.
- Simoncini T, Hafezi-Moghadam A, Brazil DP, et al. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature*. 2000;407:538–541.
- Haynes MP, Sinha D, Russell KS, et al. Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. *Circ Res*. 2000;87:677–682.
- Pervin S, Singh R, Rosenfeld ME, et al. Estradiol suppresses MCP-1 expression in vivo: implications for atherosclerosis. *Arterioscler Thromb Vasc Biol*. 1998;18:1575–1582.
- Hulley S, Grady D, Bush T, et al. HERS: randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. *JAMA*. 1998;280:605–613.
- Herrington DM, Reboussin DM, Brosnihan KB, et al. Effects of estrogen replacement on the progression of coronary-artery atherosclerosis. *N Engl J Med*. 2000;343:522–529.

Estrogen Receptor–Mediated, Nitric Oxide–Dependent Modulation of the Immunologic Barrier Function of the Endothelium: Regulation of Fas Ligand Expression by Estradiol
Carole Amant, Pernille Holm, Shu-hua Xu, Neil Tritman, Marianne Kearney and Douglas W. Losordo

Circulation. 2001;104:2576-2581

doi: 10.1161/hc4601.099406

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2001 American Heart Association, Inc. All rights reserved.

Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://circ.ahajournals.org/content/104/21/2576>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:
<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Circulation* is online at:
<http://circ.ahajournals.org/subscriptions/>