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### Research Article

The present study was designed to test the hypothesis that estrogen receptors (ER) in the blood vessel wall play a role in the modulation of the release of endothelium-derived nitric oxide (EDNO). Both basal and stimulated release of EDNO were determined in aortic rings isolated from female and male wild-type and male homozygous estrogen receptor knock-out (ERKO) mice. <sup>125</sup>I-17β-estradiol binding in aortic tissue showed significantly more high affinity cytosolic- nuclear-binding sites in male compared with female wildtype mice. Estrogen receptor transcripts were present in the aorta of male wild-type mice, but they were absent in male ERKO animals. Basal release of EDNO (determined by endothelium-dependent contraction caused by NG-nitro-arginine) was significantly higher in aorta of wild-type male mice compared with wild-type female mice, and significantly lower in the aorta of male ERKO compared with male wild-type mice. Acetylcholine-induced endothelium-dependent relaxation was similar in all groups studied. No difference was observed in the activity of calcium-dependent nitric oxide synthase in homogenates of lungs and brain taken from male wild-type and ERKO mice. These studies show a significant association between the number of estrogen receptors and basal release of EDNO in the aorta of mice, and suggest that decreased vascular estrogen receptor number may represent a novel risk factor for cardiovascular diseases.

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# Vascular Estrogen Receptors and Endothelium-derived Nitric Oxide Production in the Mouse Aorta

## Gender Difference and Effect of Estrogen Receptor Gene Disruption

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### Abstract

The present study was designed to test the hypothesis that estrogen receptors (ER) in the blood vessel wall play a role in the modulation of the release of endothelium-derived nitric oxide (EDNO). Both basal and stimulated release of EDNO were determined in aortic rings isolated from female and male wild-type and male homozygous estrogen receptor knock-out (ERKO) mice. <sup>125</sup>I-17 $\beta$ -estradiol binding in aortic tissue showed significantly more high affinity cytosolic-nuclear-binding sites in male compared with female wild-type mice. Estrogen receptor transcripts were present in the aorta of male wild-type mice, but they were absent in male ERKO animals. Basal release of EDNO (determined by endothelium-dependent contraction caused by N<sup>G</sup>-nitro-L-arginine) was significantly higher in aorta of wild-type male mice compared with wild-type female mice, and significantly lower in the aorta of male ERKO compared with male wild-type mice. Acetylcholine-induced endothelium-dependent relaxation was similar in all groups studied. No difference was observed in the activity of calcium-dependent nitric oxide synthase in homogenates of lungs and brain taken from male wild-type and ERKO mice. These studies show a significant association between the number of estrogen receptors and basal release of EDNO in the aorta of mice, and suggest that decreased vascular estrogen receptor number may represent a novel risk factor for cardiovascular diseases. (*J. Clin. Invest.* 1997. 99:2429–2437.)  
Key words: estrogen receptor • ERKO mice • <sup>125</sup>I-17 $\beta$ -estradiol binding • gender difference • nitric oxide synthase

### Introduction

Several observations indicate that the ovarian sex steroid hormone, 17 $\beta$ -estradiol, causes vasodilation and increased blood flow to various organs and provides significant protection against cardiovascular diseases, such as hypertension and atherosclerosis in animals and humans (1–5).

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The precise mechanism of estrogen-induced vasodilation and protection against atherosclerosis is not known. After the original observation by Gisclard et al. (6) several studies demonstrated enhanced release of endothelium-derived nitric oxide (EDNO) in rabbit (7) and rat aorta (8) and monkey coronary artery (9), and increased expression and/or activity of constitutive nitric oxide synthase (cNOS) in 17 $\beta$ -estradiol-treated cultured endothelial cells (10), in pregnant guinea pigs (11), and rats (12). These studies suggested that estrogen induced augmentation of NO production by the vascular endothelium may contribute to its beneficial cardiovascular actions (13). Increased endothelium-dependent coronary artery vasodilation (14) and elevated plasma levels of nitrate, the stable metabolite of NO, in postmenopausal women treated with estrogen (15) suggest that the ovarian sex hormone has a similar action in women. However, there are no reports on similar action of estrogen in males. It is also unknown whether this effect of estrogen is mediated by vascular estrogen receptors in vivo.

Estrogen receptors (ER) are present in the blood vessel wall (16–19) and a recent study showed decrease/absence of estrogen receptors in human atherosclerotic plaques (20). The availability of homozygous estrogen receptor mutant mice, where the ER gene was disrupted and which as such lack functional ER (21–23) allowed us, for the first time, to study under physiological conditions the importance of ER in the modulation of EDNO production.

In the present study we analyzed gender difference in estrogen binding and EDNO production in aorta of male and female control mice. In addition, we compared endothelium-dependent and -independent vascular responses in aortic rings and calcium-dependent and -independent nitric oxide synthase (NOS) activity in lungs and brains isolated from male wild-type and estrogen receptor knockout (ERKO) mice. The results show that aortae isolated from male wild-type mice have significantly more high affinity estradiol binding sites and produce more endothelium-derived NO than aortae from female mice. Furthermore, disruption of estrogen receptor gene causes significant reduction in basal endothelial NO production in the aorta of male ERKO mice compared with wild-type mice. These findings indicate that functional estrogen receptors in the blood vessel wall play an important role in the regulation of endothelial NO production.

1. *Abbreviations used in this paper:* Ach, acetylcholine; ecNOS, endothelial-constitutive nitric oxide synthase; EDNO, endothelial-derived nitric oxide; ER, estrogen receptor; ERKO, estrogen receptor knock-out (mice); iNOS, inducible NOS; L-NAME, N<sup>G</sup>-L-arginine methyl-ester; L-NNA, N<sup>G</sup>-nitro-L-arginine, NTG, nitroglycerin, RT-PCR, reverse transcriptase PCR; U46619, thromboxane analogue.

## Methods

### Animals

Wild-type male and female mice (C57Bl/6J) and homozygous estrogen receptor mutant (ERKO) male mice were used in this study. Development of the ERKO mouse was accomplished by homologous recombination and insertion of a neomycin sequence containing premature stop codons and polyadenylation sequences into a NotI site in exon 2 of the mouse estrogen receptor gene (21–23). In brief, targeting of the ER gene occurred by homologous recombination in 129 SV embryonic stem cell clones using double selection screening. A positive clone of ES cells was inserted into C57Bl/6J blastocysts, which were implanted into pseudopregnant hosts of the same strain mice to produce chimera. The heterozygous male offsprings, containing one copy of the wild-type ER gene and one copy of the disrupted gene (verified by Southern blot and polymerase chain reaction) (21) were fertile and exhibited no known phenotypic alteration compared to their wild-type parents. Crosses of the heterozygous mice resulted in ER homozygous mutant offsprings. The male ERKO mice (used in the present study) were infertile but otherwise perfectly viable.

### Plasma 17 $\beta$ -estradiol level determination

In wild-type ( $n = 3$ ) and ERKO ( $n = 3$ ) male mice plasma 17 $\beta$ -estradiol level was determined at the age of 10 wk by radioimmunoassay in the laboratory of K. Korach (23). Plasma 17 $\beta$ -estradiol level in control female mice ( $n = 4$ ) was determined by the Endocrinology Laboratory at Cornell University, Ithaca, NY.

### <sup>125</sup>I-17 $\beta$ -Estradiol binding in aortic tissue

Aortas from 50 C57Bl/6J male and 50 female mice were isolated and immediately frozen in liquid nitrogen. The aortas were homogenized in TEGM buffer (10 mM Tris, 1.5 mM EDTA, 10% glycerol, 3 mM MgCl<sub>2</sub>, 3 mM EGTA) with protease inhibitors (50  $\mu$ g/ml each of leupeptin, antipain, soybean trypsin inhibitor, and chymostatin) using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). Homogenate was filtered through Nitex (Tetco, Inc., Basel, Switzerland), then centrifuged to pellet nuclei at 2,500 g. Supernatant was spun at 5,000 g for 50 min to clear the cytosol, which was then used in the binding assay. Nuclei were washed twice and resuspended in homogenization buffer for assay. <sup>125</sup>I-17 $\beta$ -Estradiol (New England Nuclear, Boston, MA) binding was determined using methods previously described (24, 25) with the following modification: instead of dissolving and counting the hydroxyapatite pellet in scintillation fluid, tubes containing drained hydroxyapatite pellets were counted in a gamma counter. Scatchard plot analysis of binding data was performed using Accufit Saturation Two Site software (Lundon Software, Chagrin Falls, OH).

### RNA isolation and reverse transcriptase (RT)-PCR analysis of estrogen receptor gene expression

Aorta of wild-type and ERKO male mice were removed and snap frozen in liquid nitrogen immediately after sacrifice. Poly-A RNA isolation was carried out using the Micro-FastTrack Kit (Invitrogen Corp., San Diego, CA) according to the manufacturer's protocol.

Reverse transcriptase generation of cDNA was performed on 0.3  $\mu$ g of poly A RNA using the GeneAmp RNA PCR kit (Perkin Elmer Corp., Norwalk, CT) with random hexamers according to the manufacturer's protocol except that all reagents were scaled up to 50  $\mu$ l per reaction. PCR was carried out on 30  $\mu$ l of the reverse transcriptase generated cDNA using the following mouse ER primers as previously described (21, 23): forward 5'-ATTCCCTTCCTCCGTCTT-3' (bp 34–51) to reverse 5'-TTCCTTCTCGTTACTGC-3' (bp 702–719) (numbers in parentheses refer to the location in the mouse ER cDNA according to the GCG accession No. M38651).

### Studies with isolated aorta

Thoracic aortae from female and male wild-type and ERKO male mice were dissected, cleaned of fat and connective tissue, and placed

in physiological salt solution (PSS) of the following composition (mM): Na<sup>+</sup> 141, Cl<sup>-</sup> 125, Ca<sup>2+</sup> 2.5, K<sup>+</sup> 4.7, Mg<sup>2+</sup> 0.76, H<sub>2</sub>PO<sub>4</sub><sup>-</sup> 1.7, HCO<sub>3</sub><sup>-</sup> 25, tetrasodium EDTA 0.026, glucose 11. In some rings the endothelium was removed by placing a piece of wire in the lumen and rolling the ring gently over a wet blotting paper. The rings were mounted in a Mulvany-Halpern wire myograph (26) with circulating PSS gassed with 95% O<sub>2</sub> + 5% CO<sub>2</sub> (pH: 7.4) and kept at 37°C. Determination of optimal resting tension was carried out by construction of a length-tension curve using 80 mM KCl. Tension was increased in steps of 100 mg until no further increase in contraction was observed. The optimum resting tension was 500 mg which was applied in all subsequent experiments. The concentration of the thromboxane analog, U-46619, which caused half maximal contraction was also determined (EC<sub>50</sub> = 10<sup>-8</sup> M) and used for the analysis of basal and stimulated NO release.

**Basal release of NO.** Aortic rings with or without intact endothelium from either control or ERKO mice were mounted in the Mulvany-Halpern myograph and contracted with U46619 (10<sup>-8</sup> M) in the presence of ibuprofen (10<sup>-5</sup> M). The presence/absence of functional endothelium was tested by acetylcholine (ACh; 10<sup>-6</sup> M). Basal release of NO was estimated by N<sup>G</sup>-L-arginine (L-NNA) (10<sup>-4</sup> M)-induced endothelium-dependent facilitation of U46619 (10<sup>-8</sup> M) contraction.

**Response to acetylcholine and nitroglycerin.** Aortic rings from male wild-type and ERKO mice were stretched to 500 mg optimum passive tension and maintained at this tension throughout the experiment. After a maintained contraction to U46619 (10<sup>-8</sup> M) was obtained, ACh at cumulative doses (3  $\times$  10<sup>-9</sup>–3  $\times$  10<sup>-5</sup> M) was added to the bath. To assess the ability of the tissue to relax to a nitrovasodilator, nitroglycerin (NTG) at cumulative doses (3  $\times$  10<sup>-9</sup>–3  $\times$  10<sup>-6</sup> M) was added to the tissue bath using aortic rings with intact endothelium. In both cases, the next higher dose was added only after the response to the lower dose reached steady state (~10 min).

### Nitric oxide synthase measurement

Lung, cerebrum and cerebellum were isolated from male wild-type and ERKO mice and assayed for NOS according to the method of Bredt and Snyder (27) with slight modifications. Briefly, tissues were homogenized on ice in 10 vol of homogenizing buffer (composition in mM): Tris HCl 50, EDTA 0.1, Pefabloc 1 and dithiothreitol 1. A 50- $\mu$ l sample of the homogenate was incubated with 50  $\mu$ l of incubation buffer for 20 min at 37°C. The incubation buffer contained the following cofactors: L-arginine 10  $\mu$ M, [<sup>3</sup>H]L-arginine 5 pmol, NADPH 1 mM, calmodulin 30 nM, tetrahydrobiopterin 5  $\mu$ M, calcium 2 mM, L-valine 60 mM in a Tris buffer at a pH of 7.4. To determine calcium-independent NOS activity a buffer replacing the calcium with EGTA (5 mM) was used. Nonspecific conversion of arginine to citrulline was determined in the absence of NADPH and a separate buffer containing N<sup>G</sup>-L-arginine methylester (L-NAME) (1 mM) provided further evidence that NOS was being measured.

After the 20-min incubation the reaction was stopped by the addition of 1 ml of ice cold stop buffer (containing Hepes 20 mM, EGTA 2 mM, and EDTA 2 mM at pH 5.5). The reaction mixture was applied to 1-ml Dowex columns (Na<sup>+</sup> form, 50 W, mesh size 100–200) which had previously been equilibrated with 1 ml of stop buffer. The eluate was collected and the columns washed twice with 0.75 ml deionized water to retrieve any residual activity. A 400- $\mu$ l sample of the eluate was taken for scintillation counting.

The protein concentration of the homogenates was determined by the Bradford assay (28) using bovine serum albumin (Fraction V) as a standard.

### RT-PCR analysis of inducible nitric oxide synthase

Total RNA was isolated from pooled thoracic aortas of six ERKO and six wild-type (C57Bl/6J) male mice by the one step method of Chomczynski (29) using Ultraspec RNA reagent (Biotecx, Houston, TX). Positive control total RNA was prepared by inducing iNOS mRNA expression in the mouse macrophage cell line RAW 264.5.

Briefly, cells were plated at  $3 \times 10^6$  per 100 mm dish in DMEM (Gibco BRL, Gaithersburg, MD) with 10% FBS (Hyclone, Logan, UT). After 24 h fresh media was added containing 100 U/ml recombinant mouse IFN- $\gamma$  (Genzyme, Cambridge, MA) and 10 ng/ml LPS (Sigma Chemical Co., St. Louis, MO). Cells were then incubated for 6 h and harvested for RNA isolation. After quantification, RNA was diluted to 250 ng/ml in DEPC H<sub>2</sub>O for RT-PCR. Before RT-PCR analysis, total RNA was electrophoresed on a 1% formaldehyde agarose gel to check for intact 28S and 18S ribosomal bands. RNA samples were tested for both iNOS and  $\beta$ -actin expression using the GeneAmp ThermoStable rTth reverse transcriptase RNA PCR kit (Perkin-Elmer, Branchburg, NJ). RT-PCR primer sets for iNOS and  $\beta$ -actin were obtained from Clontech (Palo Alto, CA). RT-PCR was performed according to the manufacturers protocol with the following modifications: 250 ng of each total RNA sample was amplified using 0.75  $\mu$ M of each primer set. The RT reaction for  $\beta$ -actin was performed at 60°C for 25 min followed by a two-step PCR reaction (95°C 10 s, 65°C 20 s) for 30 cycles. The RT reaction for iNOS was performed at 65°C for 30 min followed by a two-step PCR reaction (95°C 10 s, 65°C 20 s) in 2.2 MgCl<sub>2</sub> for 35 cycles. Products were visualized by running 10  $\mu$ l of 100  $\mu$ l total reaction volume on a 2% agarose/1 $\times$  TBE gel for 1.5 h at 100 V.

### Chemicals

All chemicals used were of analytical grade. The following chemicals were used: acetylcholine, nitroglycerin, L-NNA, Dowex-50W, NADPH, L-valine, L-arginine, L-NAME, Hepes, EDTA, DTT, ibuprofen, and Tris (Sigma Chemical Co.), calmodulin, tetrahydrobiopterin (Alexis, Berlin, Germany), [<sup>3</sup>H]arginine (Amersham International, Little Chalfont, United Kingdom), EGTA (Fluka, New Ulm, Germany), Pefabloc (Boehringer Mannheim, Mannheim, Germany).

### Calculation and statistical analysis

Contractions of the aortic rings are expressed as increase in isometric tension (mg). Relaxations to ACh and NTG are expressed as percent decrease of the active tension development produced by U46619. The data are expressed as mean  $\pm$  SEM. Number of experiments (*n*) represent the number of animals from which tissues were isolated. Statistical evaluation of difference between means was performed with Student's *t* test for paired and unpaired data, and ANOVA followed by Neumann test when more than two groups were compared. The difference was considered statistically significant at *P* < 0.05.

## Results

### Endothelium-dependent vascular responses in mouse aorta

To analyze gender difference and compare vascular function in wild-type and ERKO mouse aorta a quantitative assay for endothelium-dependent and -independent responsiveness of control mouse aorta had to be developed first, as this has not been described previously. The original trace depicted in Fig. 1 shows that a ring of male mouse aorta with intact endothelium, contracted with U46619 ( $10^{-8}$  M) relaxed in response to ACh ( $10^{-6}$  M). The L-arginine analogue *N*<sup>G</sup>-L-nitro-arginine (L-NNA;  $10^{-4}$  M) caused further contraction and inhibited ACh-induced relaxation (Fig. 1, upper trace). Mechanical removal of the endothelium facilitated contraction by U46619 ( $10^{-8}$  M), abolished both L-NNA-induced contraction and ACh-induced relaxation, but had no effect on NTG-induced vasorelaxation (Fig. 1, lower trace). The mean values of six experiments demonstrate endothelium-dependent contractions to L-NNA (Fig. 2), which was used to assess basal release of EDNO.

### Gender difference

**Plasma 17 $\beta$ -estradiol level.** Circulating plasma level of 17 $\beta$ -estradiol was similar in male ERKO ( $12.9 \pm 3.4$  pg/ml; *n* = 3)

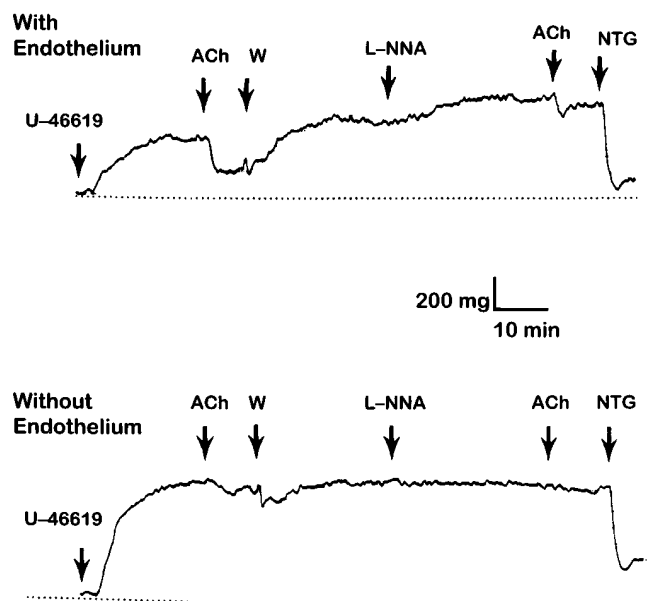


Figure 1. Endothelial function in mouse aorta. Original recording demonstrating endothelium-dependent relaxation to ACh ( $10^{-6}$  M), endothelium-dependent contraction to L-NNA ( $10^{-4}$  M), and endothelium-independent relaxation to NTG ( $10^{-6}$  M) in aortic rings from control mice (top) with and (bottom) without intact endothelium following contraction with U-46619 ( $10^{-8}$  M). W, wash out.

and wild-type mice ( $11.8 \pm 3.4$  pg/ml; *n* = 3). Female control mice had significantly higher plasma 17 $\beta$ -estradiol level than males ( $79.0 \pm 14.6$  pg/ml; *P* < 0.05; *n* = 4).

**<sup>125</sup>I-17 $\beta$  Estradiol binding in aortic tissue.** Aortic tissue isolated from both female and male wild-type mice exhibited high affinity estrogen binding in the cytosol and nucleus (Table I). The binding affinity (*K*<sub>d</sub>) was somewhat higher in the nucleus in both genders, but it was similar to the high affinity binding

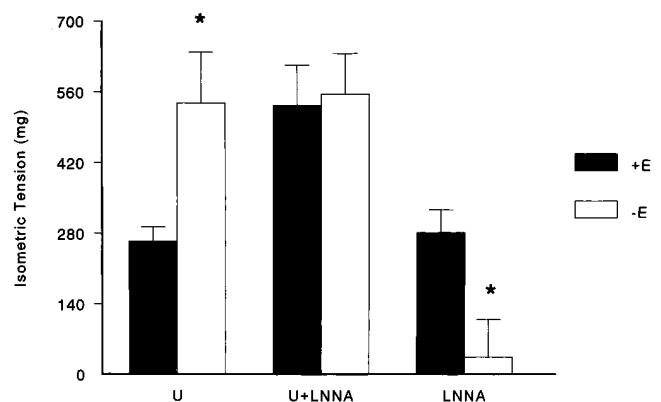


Figure 2. Effect of L-NNA on U-46619 contraction in mouse aorta. Assessment of basal endothelial nitric oxide production by endothelium-dependent contraction to *N*<sup>G</sup>-nitro-L-arginine (L-NNA;  $10^{-4}$  M), in aortic rings with (filled columns) and without endothelium (open columns) isolated from control male mice. Removal of endothelium significantly (*\*P* < 0.05) augments contractions evoked by  $10^{-8}$  M U-46619 (U) (left) and prevents contractions in response to L-NNA (middle and right). Data are shown as mean  $\pm$  SEM of six experiments.

Table I. Estrogen Receptor Levels in Mouse Aorta\*

		$K_d$	$B_{max}$
		nM	fmol/100 $\mu$ g DNA
Male	Cytosol	2.9	119
	Nuclear	1.3	142
Female	Cytosol	3.0	62
	Nuclear	1.3	47

\*Values were obtained from Scatchard plot analysis of  $^{125}$ I-17 $\beta$ -estradiol binding in aortic tissues pooled from 50 male and 50 female mice.

sites in the mouse uterus (24). In contrast to the similar binding affinity, aorta from male mice contained more than twice as many binding sites both in the cytosol and the nucleus than aorta from female mice (Table I).

**Basal and stimulated release of endothelium-derived nitric oxide.** Endothelium-dependent contractions evoked by L-NNA ( $10^{-4}$  M) (measure of basal endothelial NO production; see Figs. 1 and 2) were significantly higher in male ( $n = 6$ ) than in female ( $n = 6$ ) control mouse aorta (Fig. 3 A). No significant difference was observed in acetylcholine-induced endothelium-dependent relaxation between the two groups (Fig. 3 B).

#### Comparison of wild-type and ERKO male mice

**Estrogen receptor gene expression in the aorta.** RT-PCR analysis revealed the presence of wild-type estrogen receptor message in the aorta of wild-type mice. No wild-type estrogen receptor mRNA was detected in ERKO aorta. Variant ER transcripts originating from the disrupted ER gene were seen in ERKO mouse aorta as previously observed in the uterus (23). Results from the wild-type lanes clearly indicated expression of estrogen receptor in aortic tissues (Fig. 4).

**Basal and stimulated endothelium-derived nitric oxide production.** Vascular reactivity of aortic rings isolated from male wild-type ( $n = 6$ ) and ERKO mice ( $n = 5$ ) was compared. The passive stretch (length)-active tension relationship was identical in wild-type and ERKO mice (optimum passive tension:

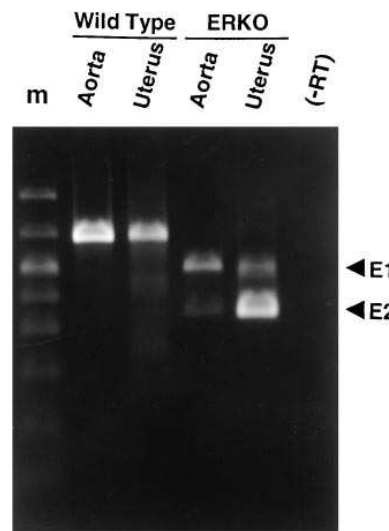


Figure 4. RT-PCR analysis for ERKO ER splicing variants E1 and E2. Poly A RNA from aorta of wild-type (WT) and ERKO male mice and uterine RNA from WT and ERKO female mice was analyzed using ER exon specific primers flanking the site of the disruption in the mouse ER gene. Uterine tissue was analyzed as a positive control for expression of the different ER variants (24). RT-PCR on male aorta and uterine RNA of wild-type mice ampli-

fies the expected 685-bp product whereas PCR on RNA from ERKO mice demonstrates the lack of wild-type ER RNA but amplifies the specific ERKO ER splicing variants E1 (514 bp) and E2 (354 bp), as previously described (24). PCR on a minus reverse-transcriptase (-RT) control was carried out on wild-type aorta RNA to rule out PCR from genomic DNA. Markers (m) correspond to DNA fragments of 1,000, 700, 525, 500, 400, 300, 200, 100, and 50 bp.

$\sim 500$  mg; Fig. 5 A). Similarly, contractions evoked by U46619 in endothelium-denuded aortic rings were similar in the two groups (Fig. 5 B).

Endothelium-dependent contractions to L-NNA ( $10^{-4}$  M) were significantly greater in wild-type ( $n = 6$ ) than in ERKO ( $n = 5$ ) aortic rings (Fig. 6). In contrast, acetylcholine-induced endothelium-dependent relaxations (inhibited by treatment of the rings with L-NNA;  $n = 5$ ; not shown) were similar in the two groups (Fig. 7 A). Nitroglycerin-induced endothelium-independent relaxations were not significantly different in the two groups (Fig. 7 B).

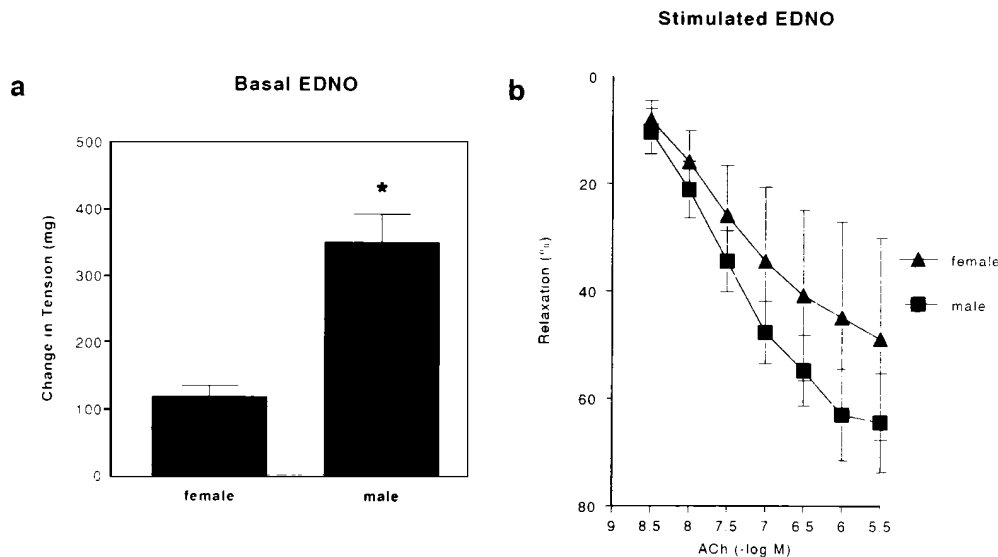
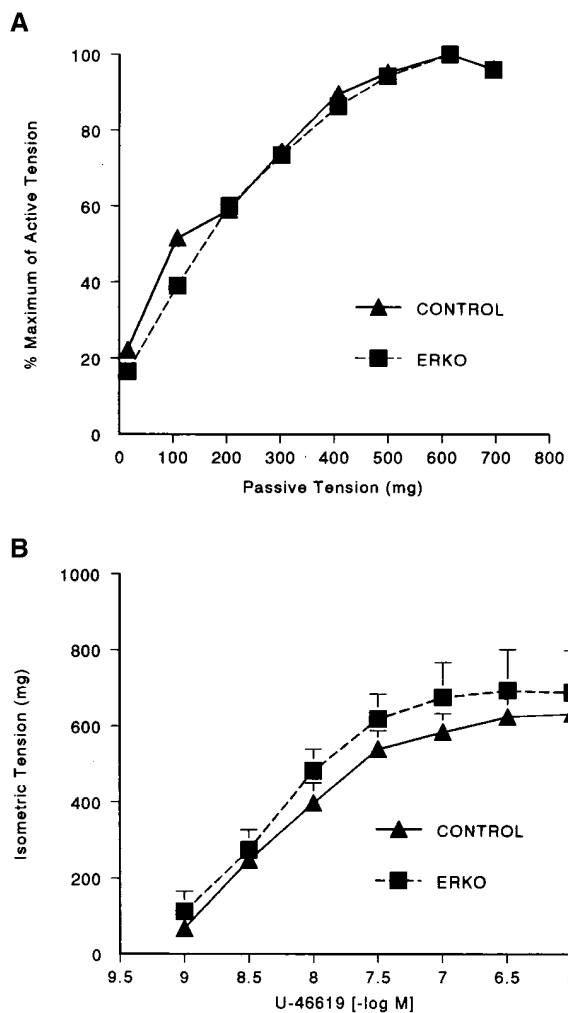


Figure 3. Comparison of (a) endothelium-dependent facilitation of U-46619 ( $10^{-4}$  M)-induced contraction by L-NNA ( $10^{-4}$  M) (basal release of EDNO) and (b) endothelium-dependent relaxation to acetylcholine (stimulated release of EDNO) in aortic rings isolated from wild-type male ( $n = 6$ ) and female ( $n = 6$ ) mice. Data are shown as mean  $\pm$  SEM of six experiments. \*Statistically significant ( $P < 0.05$ ) difference between male and female groups.



**Figure 5.** Comparison of (A) length-tension curve (top) and (B) contraction to U-46619 (bottom) in aortic rings isolated from wild-type and homozygous estrogen-receptor deficient (ERKO) mice. Active tension development caused by 80 mM KCl at various passive tension levels is expressed as percentage of maximum (reached in both tissues at 650 mg passive tension) and shown as the means of six experiments. Contractions to U-46619 are expressed as milligram increase in tension and shown as means  $\pm$  SEM of six experiments.

#### Nitric oxide synthase activity

The activity of calcium-dependent and calcium-independent NOS in cerebrum, cerebellum and lung tissue homogenates of wild-type and ERKO male mice are shown in Table II. No calcium-independent (i.e., inducible) NOS activity was observed in any of the tissues studied. Calcium-dependent (i.e., constitutive) NOS activity was, as expected, significantly higher in the brain than in the lung. The addition of L-NAME (3 mM) to the incubation buffer effectively inhibited NOS activity measured in both lung and brain. There was no evidence to suggest that NOS activity in the lung and brain of wild-type mice differed from that measured in ERKO mice.

#### Inducible nitric oxide synthase expression in the aorta

To examine iNOS mRNA expression in the mouse aorta, RT-PCR was performed on total RNA from pooled aorta of both control and ERKO male mice. As a positive control for iNOS

**Table II. Calcium-dependent (c-NOS) and Calcium-independent (i-NOS) Nitric Oxide Synthase Activity in Lung and Brain of Wild-Type and ERKO Male Mice**

	c-NOS*	i-NOS*	Total NOS* + L-NAME
<b>Lung (n = 4)</b>			
Wild-type	12.2 $\pm$ 5.46	2.2 $\pm$ 1.3	1.7 $\pm$ 1.5
ERKO	11.8 $\pm$ 4.17	1.3 $\pm$ 0.8	2.0 $\pm$ 1.0
<b>Cerebrum (n = 4)</b>			
Wild-type	473.4 $\pm$ 45.4	0 $\pm$ 0	3.0 $\pm$ 1.8
ERKO	487.4 $\pm$ 59.8	0 $\pm$ 0	5.4 $\pm$ 2.2
<b>Cerebellum (n = 5)</b>			
Wild-type	768.9 $\pm$ 43.3	3.2 $\pm$ 2.2	2.2 $\pm$ 0.8
ERKO	727.3 $\pm$ 47.3	1.9 $\pm$ 1.7	1.8 $\pm$ 1.0

\*NOS activity expressed as pmol citrulline/20 min per mg protein and shown as mean  $\pm$  SEM.

expression stimulated versus unstimulated RAW 264.5 mouse cells were used. In addition, RT-PCR using  $\beta$ -actin primers was performed on all RNA samples as a control for tube to tube variability and differences in RNA amount. Fig. 8A shows the results of a typical RT-PCR experiment using  $\beta$ -actin primers on control and ERKO mouse aorta RNA (lanes 2 and 3). In all RNA samples examined by  $\beta$ -actin RT-PCR, the relative expression level of  $\beta$ -actin was similar. We observed iNOS expression in the stimulated RAW 264.5 cells but not in the unstimulated cells (Fig. 8B, lanes 2 and 3). No iNOS expression was observed in either the control or ERKO mouse aorta (Fig. 8B, lanes 4 and 5).

## Discussion

### Endothelial regulation of vascular tone in mouse aorta

The present study shows that similar to blood vessels isolated from a variety of other species (30–34) the endothelium of the mouse aorta produces a vascular smooth muscle relaxing substance (endothelium-derived relaxing factor; [EDRF]) under basal (nonstimulated) conditions, which can be augmented by the endothelium-dependent vasodilator, acetylcholine. The nonselective NOS inhibitor L-NNA prevented both basal and stimulated EDRF release, suggesting that both basal and stimulated endothelium-dependent relaxations may be mediated by nitric oxide (NO). Similar to other vascular preparations, the source of NO is probably the endothelial constitutive NOS isoform (ecNOS), since selective disruption of the ecNOS gene in transgenic mice abolished endothelium-dependent relaxations in aortic rings isolated from these animals (35).

### Gender difference in estradiol binding and endothelial NO production in mouse aorta

Analysis of  $^{125}$ I-17 $\beta$ -estradiol binding in wild-type female and male mouse aorta revealed for the first time the existence of high affinity binding sites both in the cytosolic and nuclear fractions of this tissue. These binding sites are probably identical with the classical estrogen receptor, since (i) RT-PCR analysis documented the expression of the gene for this receptor in control mouse aortic tissue (see Fig. 4), and (ii) the binding affinity in mouse aorta is similar to that observed in the mouse

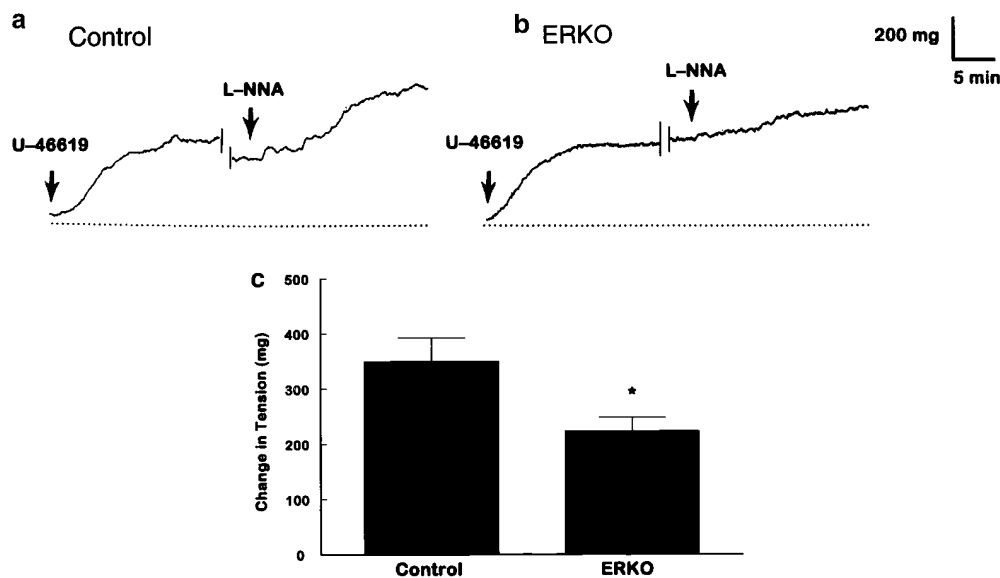


Figure 6. Endothelium-dependent contraction by L-NNA in mouse aorta. Comparison of endothelium-dependent facilitation of U-46619 ( $10^{-8}$  M)-induced contraction by LNNA ( $10^{-4}$  M) in aortic rings with endothelium isolated from wild-type and ERKO male mice: (a) original trace of a wild-type aortic ring; (b) original trace of an ERKO aortic ring; (c) mean  $\pm$  SEM of 5 (ERKO) and 6 (wild-type) experiments, respectively, expressed as percent facilitation of U-46619 contraction before L-NNA: control:  $295 \pm 33$  mg; ERKO:  $258 \pm 37$  mg).

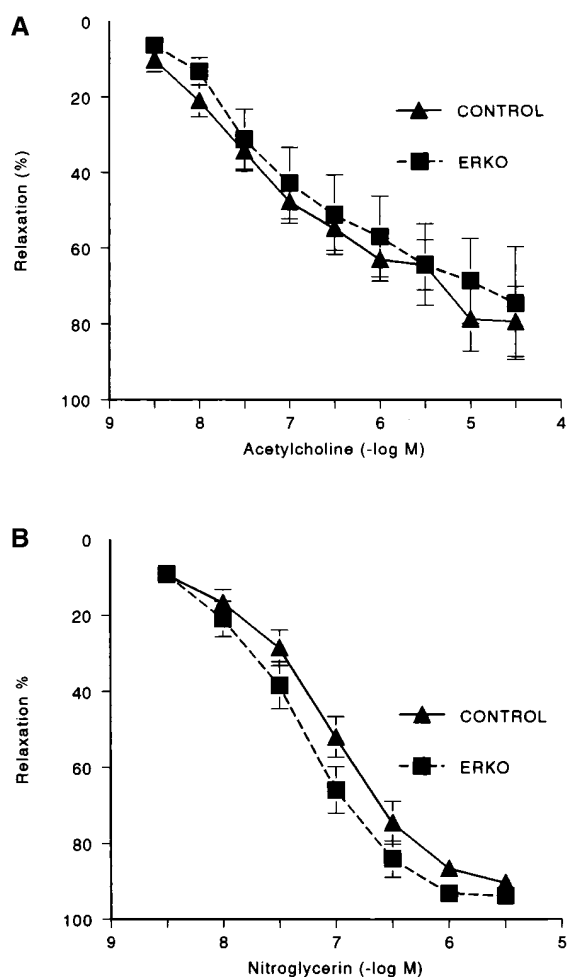


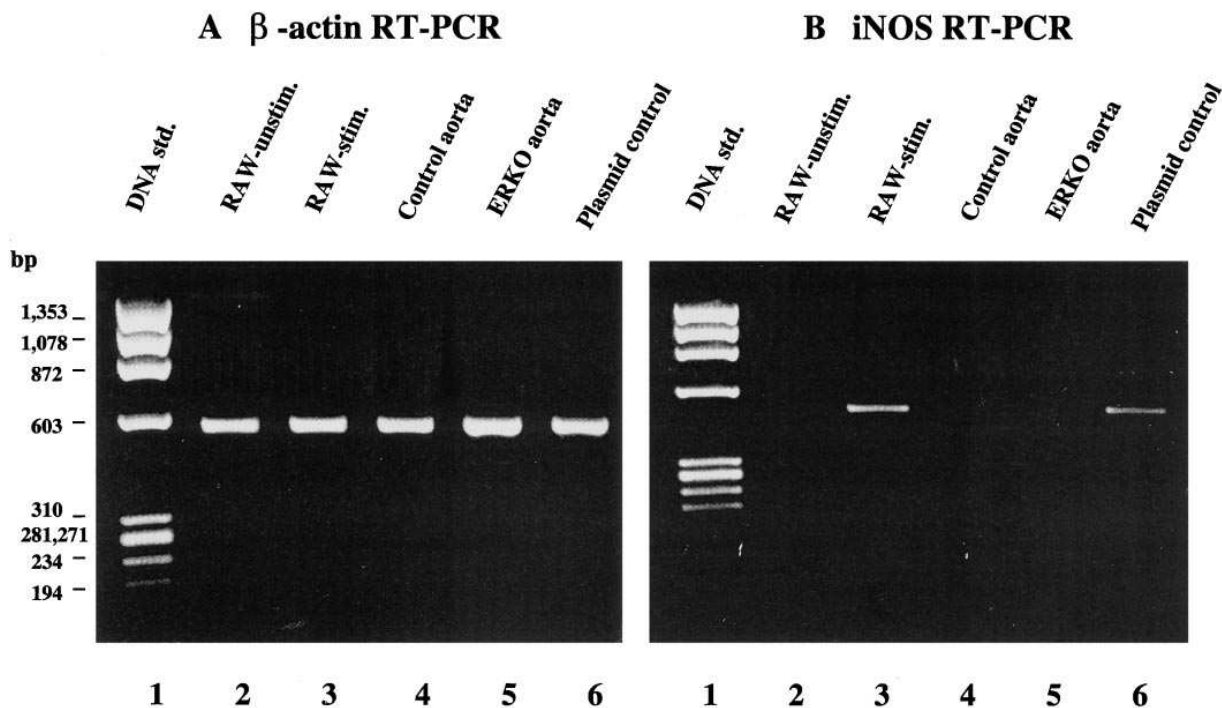
Figure 7. Concentration-response curves to (A) ACh and (B) NTG in aortic rings with intact endothelium, isolated from wild-type ( $n = 5$ ) and ERKO male mice ( $n = 5$ ), after contraction with U-46619 ( $10^{-8}$  M). Data are expressed as percent inhibition of contraction to U-46619 and shown as mean  $\pm$  SEM.

uterus (23). Comparison of estrogen binding in male and female aorta led to the unexpected discovery that although the binding affinity ( $K_d$ ) is the same (i.e., they probably contain the same receptor[s]), the number of high affinity cytosolic-nuclear estrogen binding sites is more than twice as high in male than in the female aorta (see Table I).

The present study also revealed significant gender difference in basal (but not ACh stimulated) EDNO production in wild-type mouse aorta. In contrast to earlier comparisons in rat (8) and rabbit (7), we found that the aorta from male mice produce significantly more EDNO under basal conditions than aorta from female mice. Interestingly, the higher EDNO generation was associated with higher number of estrogen binding sites in male mouse aorta, suggesting (but not proving) a causal relationship between the number of functional vascular ER and basal EDNO production. The higher EDNO production in male mice occurred in spite of significantly lower circulating levels of plasma  $17\beta$ -estradiol, when compared with female mice. This finding raises the possibility that modulation of basal EDNO production is associated primarily with the number of vascular estrogen receptors and not with the level of circulating  $17\beta$ -estradiol. Given the well documented anti-atherosclerotic properties of EDNO (see references 36 and 37), it is not surprising that male C57Bl/6J mice were reported to be more resistant to hypercholesterolemia induced atherosclerosis than female mice of this same strain (38).

#### Effect of disruption of the estrogen receptor gene

To further test the hypothesis that functional estrogen receptors in the mouse aorta modulate the level of EDNO production, we studied the effect of disruption of estrogen receptor gene on EDNO generation in male ERKO mice. We chose the male mice where higher numbers of estrogen-binding sites was associated with higher basal EDNO production (see above). The detection of the apparently low levels of ER variant in the aorta of ERKO animals is consistent with the observed phenotypic difference. Detection of the variant in ERKO tissues is even less prominent in the aorta than in the uterus where it was shown to be functionally inactive (23). The studies showed



**Figure 8.** (A) RT-PCR analysis of  $\beta$ -actin expression in male mouse aorta. Lanes: 1-DNA size standards; 2-mouse RAW264.5 cells, unstimulated; 3-mouse RAW264.5 cell stimulated with IFN- $\gamma$  and LPS; 4-control mouse aorta RNA; 5-ERKO mouse aorta RNA; 6- $\beta$ -actin RT-PCR control plasmid. (B) RT-PCR analysis of iNOS expression in male mouse aorta. Lanes: 1-DNA size standards; 2-mouse RAW264.5 cells, unstimulated; 3-mouse RAW264.5 cell stimulated with IFN- $\gamma$  and LPS; 4-control mouse aorta RNA; 5-ERKO mouse aorta RNA; 6-iNOS RT-PCR control plasmid.

that effective disruption of estrogen receptor gene leads to significant decrease in basal endothelial NO production in male ERKO mice. This change occurred in spite of unchanged circulating plasma levels of 17 $\beta$ -estradiol compared with control male mice, further supporting the hypothesis that functional estrogen receptors and not the level of circulating 17 $\beta$ -estradiol play a predominant role in modulating endothelial NO production in mouse aorta.

#### *Selective modulation of basal but not stimulated release of EDNO*

The observation that, in contrast to the endothelium-mediated contractions to L-NNA (i.e., basal NO production), endothelium-dependent relaxations to acetylcholine were not different in aorta of wild-type male and female mice or in wild-type and ERKO male mice, is in agreement with previous reports that changes in 17 $\beta$ -estradiol levels (e.g., gender difference and effects of ovariectomy and estrogen treatment) affected basal but not acetylcholine-stimulated NO release from the endothelium of rabbit (7) and rat aorta (8). Similarly, a recent study showed that estrogen-substitution of perimenopausal women significantly augmented basal (determined by the extent of vasoconstriction to L-NAME) but not ACh-stimulated EDNO production in the forearm circulation (39). The similarity between these earlier findings in females and our present observations in male mice suggest that functional ER regulates EDNO in the vasculature of both genders.

Inhibition of NO generation by L-arginine analogues (e.g., L-NNA) has been used to determine the extent of basal endothelial NO production in isolated blood vessels (32, 40) and in

animals and humans in vivo (34, 39). We used this method to compare basal EDNO production in different groups of mice.

The reduced basal EDNO production in male ERKO mouse aorta cannot be due to decreased reactivity of vascular smooth muscle cells to NO, as relaxations to the exogenous NO donor, nitroglycerin, were not different in the ERKO mouse aorta compared with control. Therefore, changes in eNOS protein (as a consequence of altered gene transcription) or activity (due to, for example, posttranslational modification and altered cofactor or substrate availability) could be the cause of the observed phenomenon.

However, decreased eNOS gene transcription and protein level should result in decreased cNOS activity in tissue homogenates (11) and decreased stimulated production of NO (10). Since no difference was observed between cNOS activity in brain and lung homogenates and ACh-stimulated EDNO release in aortic rings isolated from wild-type and ERKO mice, reduced number (control female mice) or absence of functional ER (ERKO male mice) may cause reduced basal EDNO production via alternative mechanisms.

The present findings of selective suppression of endothelium-dependent L-NNA-induced contraction but not ACh relaxation by the absence of functional ER could be explained by the following possibilities: (a) basal and stimulated NO production is controlled by different NOS enzymes, which are modulated differently by the estrogen receptor, or alternatively, (b) the same cNOS is involved, but the estrogen receptor modulates its basal activity but not its activity when stimulated by ACh in vascular rings. The possibility, that at least part of basal EDNO is generated by the inducible form of



NOS (iNOS) can be ruled out by the present finding of absence of iNOS mRNA in the aortic tissue of wild-type or ERKO male mice. Differences in NO degradation mechanism(s), and L-arginine or co-factor (e.g., tetrahydrobiopterin, NADPH, etc.) availability can be also ruled out with great probability as they would affect both basal and stimulated release/action of NO. Recent observations offer two probable, but still hypothetical, explanations for the selective modulation of basal but not stimulated endothelial NO production by ER. The first possibility assumes that functional ER leads to changes in calcium homeostasis in endothelial cells, which results in a moderate elevation of free cytosolic calcium, sufficient to increase the activity of the calcium-dependent eNOS. A recent preliminary study showed that 17 $\beta$ -estradiol elevated free cytosolic calcium in cultured bovine pulmonary artery endothelial cells via an estrogen receptor-dependent mechanism which lead to an increase of eNOS activity (41). This mechanism would also explain why ACh-stimulated NO production is not affected by gender or ER gene disruption, since significant increase in cytosolic-free calcium level accompanying the ACh response will mask the more moderate increase of calcium level produced by estrogen.

The second explanation assumes that functional ER induces posttranslational modification of eNOS which makes the enzyme produce more NO in a calcium-independent manner. Indeed, recent studies reported that both shear-stress (42) and 17 $\beta$ -estradiol (43) cause posttranslational modification of the eNOS protein in cultured human endothelial cells, resulting in elevated basal but not stimulated NO production in a Ca<sup>2+</sup>-independent manner.

Irrespective of the exact mechanism, the present study demonstrates a significant association between the number of vascular estrogen receptors and NO production by the vascular endothelium in mouse aorta. The changes in EDNO production occurred independent of the level of circulating plasma 17 $\beta$ -estradiol, suggesting that the number of vascular ER may be the primary modulator of EDNO.

A recent study showed that the number of estrogen receptors was significantly reduced in atherosclerotic plaques compared to "normal" segments of human coronary arteries (20). In light of the present findings one may speculate that a decrease or absence of estrogen receptors in the blood vessel wall may represent a novel risk factor for the development and progression of atherosclerosis. The present study also demonstrates that functional ER can modulate EDNO production not only in females but also in males. This observation may be of clinical relevance, suggesting that an appropriate estrogenic drug may be of therapeutic benefit in treating or preventing cardiovascular disease in male patients as well.

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