Estrogen-Induced Vasoprotection Is Independent of Inducible Nitric Oxide Synthase Expression
Evidence From the Mouse Carotid Artery Ligation Model

Todd Tolbert, MD; J. Anthony Thompson, PhD; Philippe Bouchard; Suzanne Oparil, MD

Background—Estrogen is vasoprotective in animal models of vascular injury, yet the mechanisms involved are incompletely understood. The role of inducible nitric oxide synthase (iNOS) in vascular repair is controversial, but many lines of evidence indicate that it plays a role in neointima formation after arterial injury and that 17β-estradiol (E₂) modulates iNOS expression. This study tested the hypothesis that E₂ reduces neointima formation after vascular injury via a mechanism that is dependent on modulation of iNOS expression.

Methods and Results—Male and female wild-type (iNOS⁻/⁻) mice and mice with homozygous deletion of the iNOS gene (iNOS⁻/⁻) were studied intact (INT) or after ovariectomy (OVX) and implantation of E₂ or vehicle (V) pellets. Mice were randomized to 8 groups based on sex, iNOS status, OVX, and treatment with E₂ or V. Twenty-eight days after carotid artery ligation, mice were euthanized, and occluded vessels were evaluated for neointima formation by morphometric analysis. There was a marked sexual dimorphism in neointima formation in both the iNOS⁻/⁻ mice and the iNOS⁻/⁻ INT females. iNOS⁻/⁻ INT females had a ≥90% reduction in neointima formation compared with iNOS⁻/⁻ males, and iNOS⁻/⁻ INT females had a 65% reduction in neointima formation compared with iNOS⁻/⁻ males. The sexually dimorphic response was attenuated by OVX and restored by E₂ replacement in both iNOS⁻/⁻ and iNOS⁻/⁻ mice.

Conclusions—These results demonstrate that the vasoprotective effects of E₂ after ligation vascular injury are, at least in part, independent of iNOS expression. (Circulation. 2001;104:2740-2745.)

Key Words: hormones ■ vasculature ■ nitric oxide synthase

There is an abundance of clinical and epidemiological evidence of a sexually dimorphic pattern of atherosclerotic cardiovascular disease in humans that has been attributed to the vasoprotective effects of estrogen. The low incidence of cardiovascular disease events among premenopausal women compared with age-matched men is well recognized, and there is a strong link between menopause and an increased incidence of cardiovascular disease in women.¹ Observational studies suggest that postmenopausal estrogen replacement therapy reduces cardiovascular disease risk by approximate 50%.²-⁴ The vasoprotective effects of estrogen have been attributed to a combination of alterations in lipid metabolism and, more importantly, direct vascular actions, including improved endothelial function and vasorelaxation and reductions in smooth muscle cell proliferation and associated extracellular matrix formation in response to vascular injury.⁵,⁶ Endothelial cells and vascular smooth muscle cells express estrogen receptors and thus possess the potential for transcriptional regulation of target genes by estrogen.⁷,⁸ Understanding of the cellular and molecular mechanisms by which estrogen exerts its favorable effects, however, is incomplete. One component of the vascular injury response that can be modulated by estrogen involves altered production of nitric oxide (·NO) due to modulation of nitric oxide synthase (NOS). The synthesis of ·NO, a potent endogenous vasodilator, is regulated by 2 major isoforms of NOS. Endothelial NOS (eNOS, NOS III) is constitutively expressed in endothelial cells and is upregulated by estrogen via an estrogen receptor–mediated mechanism.⁹ ·NO produced by eNOS plays a vasoprotective role after vascular injury, in part by inhibiting neointima formation.¹⁰ Inducible NOS (iNOS, NOS II) is not found in normal blood vessels but is expressed in injured arteries within 1 day of the insult.¹¹ Once activated, iNOS can produce >1000-fold more ·NO (micromolar range) for a longer duration than eNOS.¹² At these concentrations, ·NO can be toxic to tissues via interaction with reactive oxygen species to produce powerful biological oxidants that have been detected in human atherosclerotic lesions.¹³,¹⁴ High levels of ·NO that result from expression of iNOS have been implicated in the formation of neointima after vascular injury.¹⁵ Estrogen has directionally opposite effects on the 2 isoforms of NOS that are expressed in blood vessel

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walls: it enhances eNOS expression and inhibits iNOS expression in a variety of in vitro and in vivo preparations.16–20

The purpose of the present study was to assess the role of iNOS expression in estrogen-mediated vasoprotection after ligation vascular injury in animals of defined genotype. We hypothesized that estrogen reduces neointima formation after vascular injury via a mechanism that is dependent on modulation of iNOS expression. The results presented here demonstrate that, after carotid artery ligation, neointima formation is reduced in the presence of estrogen via a mechanism that is, at least in part, independent of iNOS expression.

Methods

Animals

Male and female wild-type (iNOS+/-) mice and mice with homozygous deletion of the iNOS gene (iNOS-/-) were studied (C57Bl/6/J background; 10 weeks old; Jackson Laboratory, Bar Harbor, Me). All mice were maintained at constant humidity (60±5%), temperature (24±1°C), and light cycle (6 AM to 6 PM) and were fed a standard mouse pellet diet (Ralston Purina Diet) ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85-23, revised 1985). Males were studied intact (INT), and females were studied after gonadectomy (OVX) and treatment with E2 or V.

Carotid Ligation Injury

Mice were anesthetized with ketamine (80 mg/kg IV; Abbott Laboratories) and xylazine (5 mg/kg IV; Rompun, Bayer Corp). Under a dissecting microscope, the right common carotid artery was exposed through a midline cervical incision and ligated with an 8-0 silk suture just proximal to the bifurcation, as previously described.21 The left common carotid artery was not injured and served as a control.

Morphometric Analysis

Mice were euthanized 28 days after injury with an overdose of pentobarbital. The vascular system was flushed with 0.1 mol/L sodium phosphate buffer (pH 7.3), followed by perfusion with 4% paraformaldehyde. Both carotid arteries were excised, fixed in 4% paraformaldehyde, and embedded in paraffin. Serial sections of occluded vessels were obtained at 200-μm intervals beginning 500 μm proximal to the ligation. Serial sections of nonoccluded control vessels were obtained in a similar manner. Slides were stained with hematoxylin and eosin and/or Verhoeff’s elastic stain, which demonstrated several layers of elastic lamina. Computer-assisted morphometric analysis of digitized images from each arterial segment was performed with image analysis software (Scion Image). Measurements of 3 serial sections (500, 700, and 900 μm proximal to the ligation) from each vessel were averaged for statistical analysis. The cross-sectional surface area of the vessel between the external elastic lamina and internal elastic lamina (medial area) and the area between the internal elastic lamina and the lumen (intimal area) were measured. The degree of neointima formation in the occluded carotid artery was expressed as the absolute area of neointima and the intima/media ratio.

Estrogen Assay

At the time the animals were killed, a 0.5-mL blood sample was removed from the right ventricle for serum E2 levels by radioimmunoassay with a commercially available kit (Coat-A-Count Estradiol; Diagnostic Products Corp) with a sensitivity of 8 pg/mL. Mice were not staged for estrus cycle at the time of sample collection.

Statistical Analysis

Results are expressed as mean±SEM. All statistical analyses were performed with computer-based statistical analysis software (Sig-maStat, Jandel Scientific). Comparisons among experimental groups were performed with 1-way ANOVA followed by pairwise multiple comparison using the Student-Newman-Keuls method. Differences were reported as significant at a value of P<0.05.

Results

The mean body weight of all male mice studied (27.3±1.3 g; n=13) was significantly greater than that of the females (20.9±0.9 g; n=53) before experimentation. No significant differences in mean body weight were noted among experimental groups based on genotype (Table).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Experimental Groups</th>
<th>Body Weight, g</th>
<th>Uterine Weight, mg</th>
<th>E2 Level, pg/mL</th>
<th>Intima:Media Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male INT</td>
<td>7</td>
<td>32.5±0.9</td>
<td>ND</td>
<td>ND</td>
<td>0.63±0.12</td>
</tr>
<tr>
<td>Female INT</td>
<td>5</td>
<td>22.4±0.4</td>
<td>ND</td>
<td>ND</td>
<td>0.08±0.02*</td>
</tr>
<tr>
<td>OVX + V</td>
<td>7</td>
<td>23.1±0.9</td>
<td>ND</td>
<td>ND</td>
<td>0.58±0.09</td>
</tr>
<tr>
<td>Male OVX</td>
<td>7</td>
<td>22.7±0.4</td>
<td>ND</td>
<td>ND</td>
<td>0.12±0.05†</td>
</tr>
<tr>
<td>Female OVX</td>
<td>8</td>
<td>30.8±0.6</td>
<td>ND</td>
<td>ND</td>
<td>0.33±0.06*</td>
</tr>
<tr>
<td>Male E2</td>
<td>7</td>
<td>21.8±0.3</td>
<td>ND</td>
<td>ND</td>
<td>0.14±0.03†</td>
</tr>
<tr>
<td>Female E2</td>
<td>8</td>
<td>22.0±0.3</td>
<td>ND</td>
<td>ND</td>
<td>0.33±0.05†</td>
</tr>
<tr>
<td>Male V</td>
<td>7</td>
<td>23.7±0.6</td>
<td>ND</td>
<td>ND</td>
<td>0.10±0.10§</td>
</tr>
<tr>
<td>Female V</td>
<td>8</td>
<td>4.61±0.45</td>
<td>ND</td>
<td>ND</td>
<td>0.32±0.04§</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SEM. ND indicates not detectable. *P<0.05 vs iNOS+/- male INT. †P<0.05 vs iNOS+/- female OVX+V. ‡P<0.05 vs iNOS-/- male INT. §P<0.05 vs iNOS-/- female OVX+V.
In a 100-μL aliquot, serum E2 concentrations could not be detected in INT or OVX+V animals. The assay was repeated with uninjured INT iNOS+/+ and iNOS−/− females (n=8 per group; results not shown), and again, serum E2 concentrations could not be detected. E2 levels were measurable in animals treated with E2 pellets (Table). Uterine weights were similar in E2-replaced females and INT females for both genotypes, whereas OVX resulted in marked reduction of uterine weight.

Both occluded right and nonoccluded left carotid arteries were examined histologically after perfusion-fixation at 28 days after injury. In the nonoccluded left carotid arteries, the intima was a single cell layer thick; the internal elastic lamina was intact, and the external elastic lamina was in contact with the adventitia in all mice examined. There were no differences in the medial areas (wall thickness) of the nonoccluded left carotid artery among experimental groups, indicating that the anatomy of the intact carotid artery was not significantly different between the sexes and was not significantly altered by E2 treatment or genotype.

Twenty-eight days after carotid ligation, occluded arteries had neointima formation consisting of circumferentially uniform layers of cells (Figure 1). Morphometric analysis of occluded vessels from iNOS+/+ mice (Figure 2 and Table) demonstrated a marked sexual dimorphism in the injury response. INT females had a >90% reduction in neointima formation compared with INT males. The sexual dimorphism was attenuated by OVX, as indicated by neointimal areas in OVX+V mice, which did not differ significantly from those of INT males. Furthermore, E2 replacement in OVX females restored the sexual dimorphism in the injury response, with OVX+E2 mice having an almost 6-fold reduction in neointima formation compared with OVX+V mice. Morphometric analysis of medial areas in iNOS+/+ mice showed that INT females had reduced medial areas compared with INT males. Medial areas, however, were not significantly different between OVX+V females and INT males or OVX+E2 females (Figure 2 and Table).

To assess the effect of iNOS expression on neointima formation after ligation vascular injury, morphometric analyses of occluded carotid arteries from iNOS−/− mice were compared with those of iNOS+/+ mice of similar sex and treatment groups (Figure 3 and Table). Compared with iNOS+/+ males, the iNOS−/− males had a >50% reduction in neointima formation. Similarly, iNOS−/− OVX+V females had reductions in neointima formation of nearly 50% compared with iNOS+/+ OVX+V females. Thus, lack of iNOS expression alone was sufficient to attenuate neointima formation 28 days after carotid artery ligation injury. No difference was noted in the extent of neointima formation between the iNOS−/− INT and the iNOS−/− INT females or between the iNOS−/− OVX+E2 and iNOS−/− OVX+E2 females. There appears to be a floor effect in the degree of neointima formation that may have masked any differences between the latter 2 groups.

Among the iNOS−/− animals, sexual dimorphism in the ligation injury response similar to that seen in the iNOS+/+ animals was demonstrated (Figure 3 and Table). iNOS−/− INT females had a 65% reduction in neointima formation compared with male iNOS−/− mice. Again, the dimorphism was attenuated by OVX, as indicated by neointimal areas in iNOS−/− OVX+V mice, which did not differ significantly from those of male iNOS−/− mice. As seen in the iNOS+/+ animals, E2 replacement in iNOS−/− OVX females restored the sexual dimorphism in the injury response, with OVX+E2 mice having a 70% reduction in neointima formation compared with OVX+V mice. Thus, E2 reduced neointima formation independently of iNOS expression and to a level comparable to that seen in iNOS+/+ mice. Medial areas did not differ significantly between the iNOS−/− mice and the iNOS−/− mice or among the various iNOS−/− groups.

**Discussion**

Our results demonstrate that (1) there is a sexually dimorphic (male>female) response in neointima formation after carotid ligation injury in the mouse; (2) OVX attenuates the sexual dimorphism of the injury response such that the neointimal area of OVX+V mice is not significantly different from that of the intact male; (3) E2 replacement dramatically reduces neointima formation in OVX mice; (4) in iNOS-deficient mice, neointima formation is attenuated after carotid ligation injury.
compared with iNOS−/− mice of similar sex and treatment group, indicating that lack of iNOS expression alone is sufficient to attenuate neointima formation; and (5) E₂ exerts a vasoprotective action by reducing neointima formation independently of iNOS expression.

The vasoprotective effects of E₂ after vascular injury are well established, but the mechanisms involved have not been thoroughly characterized. E₂ has been shown to reduce neointima formation and vascular smooth muscle cell proliferation after endoluminal wire injury of the carotid artery in mice. Previous studies from our laboratory have demonstrated an E₂-dependent sexual dimorphism in the response to balloon injury of the rat carotid artery, in which E₂ reduces neointima formation. Adding to these findings, we demonstrated that simultaneous antagonism of both the α- and β-estrogen receptor subtypes inhibited the vasoprotective effects of E₂, thus indicating that E₂ exerts its vasoprotective effects in an estrogen receptor–dependent mechanism.

In the present study, we have advanced the understanding of the mechanisms of E₂-mediated vasoprotection by examining a potential signaling pathway involving iNOS.

The most dramatic finding in this study was that E₂ reduced neointima formation in mice with homozygous deletion of the iNOS gene. Many lines of evidence indicate that induction of iNOS expression plays a role in the response to vascular injury and that E₂ modulates iNOS expression. iNOS is not found in normal blood vessels but is expressed in medial and intimal compartments of injured arteries within 1 day of the insult. Arthur and colleagues found immunohistochemical evidence of enhanced iNOS expression in the neointima of rabbit carotid arteries 7 to 14 days after placement of a periarterial Silastic collar. Colocalization of iNOS immunofluorescence with anti-smooth muscle myosin in the intima implicated vascular smooth muscle cells as the major source of iNOS in the injured vessel. Similar increases in iNOS expression in medial and neointimal vascular smooth muscle cells have been noted after balloon arterial injury in the rat and pig.

Furthermore, enhanced iNOS expression in neointima of arteries has been found in animal models of transplant arteriosclerosis as well as transplanted human hearts with accelerated graft arteriosclerosis. Thus, increased expression of iNOS clearly occurs in the course of the response to various types of vascular injury.

The functional contribution of iNOS to the vascular injury response has been assessed previously in iNOS−/− mice. In one study, Chyu and colleagues used a periadventitial arterial injury model (nonocclusive plastic cuff) in male iNOS−/− and iNOS−/− mice. As opposed to the present study and that of Chyu and colleagues, there was a trend toward reduced neointima formation. In the present study, we have demonstrated that simultaneous antagonism of both the α- and β-estrogen receptor subtypes inhibited the vasoprotective effects of E₂, thus indicating that E₂ exerts its vasoprotective effects in an estrogen receptor–dependent mechanism.

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neointima formation in iNOS−/− mice, but the reduction was not statistically significant. The eNOS−/− mice, however, had significantly more neointima formation than either iNOS+/− or iNOS−/− mice in that study. The authors also noted suppression of constrictive remodeling in the iNOS mice. Neither of these studies assessed sexual differences in the vascular injury response in iNOS-deficient mice, and neither tested the hypothesis that estrogen-mediated vasoprotection involves modulation of iNOS expression.

The effects of iNOS on neointima formation have also been examined in the setting of transplant arteriosclerosis. Koglin et al31 used iNOS−/− mice in a chronic cardiac rejection model and found significantly increased luminal occlusion and intima/media ratios in allografts from iNOS−/− mice. They concluded that iNOS plays a protective role in the development of transplant arteriosclerosis by suppressing neointimal smooth muscle cell accumulation. The observations of Koglin and colleagues differ from those seen in mechanical models of vascular injury involving reduced flow, including the present study, and suggest unique, perhaps model-specific, antiproliferative properties of iNOS in transplant arteriosclerosis.10,30

There is substantial evidence from both in vitro and in vivo studies that E2 decreases iNOS expression. Hayashi and colleagues32 demonstrated in vitro that E2 inhibits cytokine-induced iNOS expression via an estrogen receptor–mediated pathway in a murine macrophage cell line. Subsequent studies extended the in vitro evidence for a negative modulating effect of E2 on iNOS induction to rat vascular smooth muscle cells and to rat aortic endothelial cells.16–18 In vivo evidence for E2 inhibition of iNOS expression in vascular smooth muscle cells comes from experiments in rat aortic allografts and in aorta of OVX rats. Saito et al19 found decreased iNOS expression in the neointima, media, and adventitia of orthotopic abdominal aortic allografts in male rats treated with E2 compared with placebo. Tamura and colleagues20 analyzed iNOS protein expression by Western blotting in aortic homogenates from female rats studied intact and after OVX ± E2 replacement. They found that OVX rats had 2.2-fold greater aortic iNOS protein expression than intact animals and that E2 replacement attenuated iNOS expression in OVX rats. These studies provide substantial evidence that E2 reduces iNOS expression in the vasculature but do not elucidate the functional consequences of this modulating effect. We sought to advance the understanding of E2–mediated vasoprotection by examining the functional consequences of E2 administration on the response to ligation vascular injury in an animal model of controlled genotype.

In conclusion, given (1) the established vasoprotective properties of E2, (2) the association of various types of vascular injury and enhanced iNOS expression, (3) evidence of reduced neointima formation in vascular injury models with reduced flow in the absence of iNOS expression, and (4) the negative modulating effect of E2 on iNOS expression, we hypothesized that E2 exerts its vasoprotective effects via an iNOS-dependent mechanism. Our results, however, demonstrate that E2 reduced neointima formation after carotid ligation in iNOS-deficient mice to an extent similar to that seen in iNOS+/+ mice. Thus, E2 has vasoprotective actions that are, at least in part, independent of iNOS expression.

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