Clinical observations suggest that postmenopausal women have a higher incidence of aneurysmal subarachnoid hemorrhage than premenopausal women. In addition, hormone replacement regimens that contain estrogen seem to reduce the risk of subarachnoid hemorrhage in postmenopausal women. These epidemiological observations suggest the potentially protective role of estrogen against the development of aneurysmal rupture in postmenopausal women. Experimental studies using a rat model of intracranial aneurysms indicate the protective effect of estrogen against the development of aneurysms. However, no experimental study has sought to establish a direct link between estrogen and the prevention of aneurysmal rupture.

In this study, we assessed the effects of estrogen and selective estrogen receptor (ER) subtype agonists on the development of aneurysmal rupture in ovariectomized female mice. Ovariectomized female mice were used to mimic the conditions of postmenopausal women. We sought to investigate the receptor subtype and the underlying mechanisms responsible for the potentially protective effect of estrogen against the development of aneurysmal subarachnoid hemorrhage in postmenopausal women. We used an intracranial aneurysm mouse model that recapitulates the key features of human intracranial aneurysms, including spontaneous rupture. Ten- to 12-week-old ovariectomized female mice received treatment with estrogen, nonselective estrogen receptor antagonist, estrogen receptor-α agonist, or estrogen receptor-β agonist starting 6 days after aneurysm induction so that the treatments affected the development of aneurysmal rupture without affecting aneurysmal formation. Estrogen significantly reduced the incidence of ruptured aneurysms and rupture rates in ovariectomized mice. Nonselective estrogen receptor antagonist abolished the protective effect of estrogen. Although estrogen receptor-α agonist did not affect the incidence of ruptured aneurysms or rupture rates, estrogen receptor-β agonist prevented aneurysmal rupture without affecting the formation of aneurysms. The protective role of estrogen receptor-β agonist was abolished by the inhibition of nitric oxide synthase. We showed that estrogen prevented aneurysmal rupture in ovariectomized female mice. The protective effect of estrogen seemed to occur through the activation of estrogen receptor-β, a predominant subtype of estrogen receptor in human intracranial aneurysms and cerebral arteries.

Key Words: estrogens ■ menopause ■ models, animal

Abstract—Clinical observations suggest that postmenopausal women have a higher incidence of aneurysmal subarachnoid hemorrhage than premenopausal women. We hypothesize that a relative deficiency in estrogen may increase the risks of aneurysmal growth and subarachnoid hemorrhage in postmenopausal women. We assessed the effects of estrogen and selective estrogen receptor subtype agonists on the development of aneurysmal rupture in ovariectomized female mice. We used an intracranial aneurysm mouse model that recapitulates the key features of human intracranial aneurysms, including spontaneous rupture. Ten- to 12-week-old ovariectomized female mice received treatment with estrogen, nonselective estrogen receptor antagonist, estrogen receptor-α agonist, or estrogen receptor-β agonist starting 6 days after aneurysm induction so that the treatments affected the development of aneurysmal rupture without affecting aneurysmal formation. Estrogen significantly reduced the incidence of ruptured aneurysms and rupture rates in ovariectomized mice. Nonselective estrogen receptor antagonist abolished the protective effect of estrogen. Although estrogen receptor-α agonist did not affect the incidence of ruptured aneurysms or rupture rates, estrogen receptor-β agonist prevented aneurysmal rupture without affecting the formation of aneurysms. The protective role of estrogen receptor-β agonist was abolished by the inhibition of nitric oxide synthase. We showed that estrogen prevented aneurysmal rupture in ovariectomized female mice. The protective effect of estrogen seemed to occur through the activation of estrogen receptor-β, a predominant subtype of estrogen receptor in human intracranial aneurysms and cerebral arteries. (Hypertension. 2014;63:1339-1344.) • Online Data Supplement

Methods

Experiments were conducted in accordance with the guidelines approved by the University of California, San Francisco, Institutional Animal Care and Use Committee. We combined induced systemic hypertension (deoxycorticosterone acetate-salt hypertension) and a single injection of elastase into the cerebrospinal fluid at the right basal cistern, as previously described. Bilateral ovariectomy or sham ovariectomy was performed 1 week before aneurysm induction. Detailed Methods are presented in the online-only Data Supplement.
To detect aneurysmal rupture, 2 blinded observers performed daily neurological examination, as previously described. Neurological symptoms were scored as follows: 0, normal function; 1, reduced eating or drinking activity demonstrated by a weight loss >2 g of body weight (=10% weight loss) >24 hours; 2, flexion of the torso and forelimbs on lifting the whole animal by the tail; 3, circling to 1 side with a normal posture at rest; 4, leaning to 1 side at rest; and 5, no spontaneous activity. Mice were euthanized when they developed neurological symptoms (score, 1–5). All asymptomatic mice were euthanized 21 days after aneurysm induction. The brain samples were perfused with phosphate-buffered saline, followed by a gelatin containing blue dye to visualize cerebral arteries. Aneurysms were defined as a localized outward bulging of the vascular wall, whose diameter was greater than the parent artery diameter. Figure 1A, 1B, and 1C shows a representative mouse with normal cerebral arteries, an unruptured aneurysm from a mouse that was asymptomatic throughout the experimental period, and a ruptured aneurysm with subarachnoid hemorrhage from a mouse that became symptomatic 10 days after aneurysm induction, respectively.

Our previous study found that aneurysm formation occurs during the first 6 days after aneurysm induction in this model and that aneurysmal rupture begins to occur ≈7 days after the aneurysm induction. Therefore, in this study, the treatments with estrogen (17β-estradiol, 0.0017 mg/kg per day), nonselective ER antagonist (ER antagonist: Fulvestrant [ICI-182780], 3 mg/kg per day), propyl pyrazole triol (ER-α agonist: propyl pyrazole triol, 0.17 mg/kg per day), or diarylpropionitrile (ER-β agonist: DPN, 0.17 mg/kg per day) were started 6 days after aneurysm induction so that the treatments affected the development of aneurysmal rupture without affecting aneurysmal formation (Figure 1D). Dosages of estrogen and ER agonists and ER antagonist were chosen based on previous publications.

Human intracranial aneurysm and superficial temporal artery tissues were collected according to the protocol approved by the University of Iowa Institutional Review Board. Three aneurysm tissues and 1 superficial temporal artery tissue were collected and stained with anti–ER-α antibody (ID5; Dako, Carpinteria, CA) or anti–ER-β antibody (Ab3577; Abcam, Cambridge, MA). Two blinded observers counted ER-positive cells in randomly chosen areas. Semiquantitative analysis of the slides was performed based on the immunostained-positive cell counts per high-power field (×40): grade 0, 0 to 10 cells; grade 1, 10 to 20 cells; grade 2, 20 to 30 cells; grade 3, >30 cells.

Statistical Analysis
We used the Fisher exact test to analyze the incidence of ruptured intracranial aneurysms and the rupture rate (number of mice with ruptured aneurysm/number of mice with ruptured or unruptured aneurysms). As an exploratory analysis, the survival analysis was performed using the log-rank test. Mice that did not develop aneurysms were excluded from the survival analysis. All the results were expressed as the mean±SD. \( P<0.05 \) was considered statistically significant.

Results
Expression of ERs in Human Intracranial Aneurysms
We collected 3 human intracranial aneurysm tissues and 1 control artery (superficial temporal artery) from female patients who underwent aneurysm clipping. Positive control tissues exhibited strong expression of both ER-α and ER-β (Figure 2). In the control artery, ER-β was expressed in the smooth muscle layers, but ER-α was almost absent (Figure 2). Similarly, there was no significant expression of ER-α in the aneurysm wall. ER-β was expressed in the smooth muscle layers and organized thrombus of the intracranial aneurysms (Figure 2).
Effects of Estrogen on the Development of Aneurysmal Rupture in Ovariectomized Mice

To test whether estrogen is protective against the development of aneurysmal rupture in ovariectomized mice (ie, postmenopausal mice), we treated ovariectomized mice with estrogen (17β-estradiol), vehicle, or estrogen+nonselective ER antagonist (ICI-182780) starting at 6 days after aneurysm induction. The treatments were continued for 2 weeks. Treatments with estrogen or estrogen+nonselective ER antagonist started 6 days after aneurysm induction did not significantly affect the formation of aneurysms, as demonstrated by no difference in the total incidence of aneurysms (ie, the incidence of both ruptured and unruptured aneurysms) among the 3 groups (Figure 3A). However, when compared with the vehicle treatment, the estrogen treatment significantly reduced both the incidence of ruptured aneurysms (Figure 3A; vehicle control versus estrogen, 63% versus 18%; \(P<0.01\)) and the rupture rate (Figure 3B; vehicle control versus estrogen, 86% versus 23%; \(P<0.01\)) in ovariectomized mice. For the purpose of exploratory analysis, a symptom-free curve (Kaplan–Meier analysis curve) was plotted after excluding mice that did not have aneurysms (Figure 3C). A log-rank test revealed a significant reduction of aneurysmal rupture with the estrogen treatment (\(P<0.01\)).

Furthermore, the protective effect of estrogen against the development of aneurysmal rupture was abolished by...
the treatment with nonselective ER antagonist (Figure 3A and 3B; incidence of ruptured aneurysms: estrogen versus estrogen+ER antagonist, 18% versus 59%; P<0.05 and rupture rate: estrogen versus estrogen+ER antagonist, 23% versus 71%; P<0.05), confirming that the protective effect of estrogen was mediated by the activation of ERs.

**Stimulation of ER-β, but Not of ER-α, Protected Against the Development of Aneurysmal Rupture**

To identify the ER subtype that was responsible for the protective role of estrogen against the development of aneurysmal rupture, we treated the ovariectomized female mice with ER-α agonist (propyl pyrazole triol) or ER-β agonist (DPN).

Neither ER-α agonist nor ER-β agonist affected the overall incidence of aneurysms. However, the treatment with ER-β agonist significantly reduced both the incidence of ruptured aneurysms (Figure 4A; vehicle control versus estrogen receptor-β agonist, 63% versus 13%; P<0.01) and the rupture rate (Figure 4B; vehicle control versus estrogen receptor-β agonist, 86% versus 18%; P<0.01). In contrast, ER-α agonist did not have any significant effects on the aneurysmal formation or rupture (Figure 4A and 4B). Log-rank tests revealed a significant reduction of aneurysmal rupture with the ER-β agonist treatment (P<0.01) but not with the ER-α agonist treatment (Figure 4C).

**Protective Effect of ER-β Stimulation Depends on Nitric Oxide Production**

Estrogen can exert various effects on vasculature by increasing the production and availability of nitric oxide. The stimulation of ER-β by estrogen can upregulate both inducible and endothelial nitric oxide synthases, and nitric oxide causes s-nitrosylation of various target proteins that are important for tissue remodeling. Therefore, we tested whether the protective effect of ER-β stimulation against the development of aneurysmal rupture was dependent on the production of nitric oxide.

We treated mice with a nonspecific nitric oxide synthase inhibitor, N-nitro-L-arginine methyl ester (L-NAME). Although L-NAME alone did not affect the incidence of ruptured aneurysms or the rupture rate in ovariectomized mice, it abolished the protective effect of ER-β agonist (DPN; Figure 5A and 5B; incidence of ruptured aneurysms: L-NAME+DPN versus DPN alone, 69% versus 13%; P<0.01 and rupture rate: L-NAME+DPN versus DPN alone, 79% versus 18%, respectively; P<0.01), indicating that the protective effect of ER-β stimulation against the development of aneurysmal rupture depends on the production of nitric oxide by nitric synthase.

In our study, the L-NAME did not significantly affect the blood pressure. This is probably because blood pressure in our model was already raised by the deoxycorticosterone acetate-salt treatment. Deoxycorticosterone acetate-salt-induced hypertension probably masked the blood pressure augmentation effect of L-NAME that is normally observed in normotensive animals. Similarly, estrogen or ER agonist did not affect the blood pressure. Again, this is probably because of the hypertensive effect of deoxycorticosterone acetate-salt treatment that negated the effects from estrogen or ER agonist (Table S2).

**Discussion**

In this study, we showed that estrogen prevented aneurysmal rupture in ovariectomized female mice, consistent with the epidemiological studies. The protective effect of estrogen seemed to occur through the activation of ER-β, a predominant subtype of ER in human intracranial aneurysms and cerebral arteries. Furthermore, the protective effect of ER-β stimulation was dependent on the production of nitric oxide. These findings support the causal and mechanistic link between the stimulation of ER-β by estrogen and the prevention of aneurysmal rupture.

ER-α and ER-β regulate different sets of genes and mediate different cellular and tissue effects. For example, in vascular smooth muscle cells, ER-β mediates the upregulation of nitric oxide synthase, but ER-α exerts the opposite effect. We found that ER-β is a predominant form of ER in human intracranial aneurysms and superficial temporal artery samples, leading us to speculate that estrogen’s effects on cerebral arteries and intracranial aneurysms are mainly mediated by ER-β.

**Figure 4.** Stimulation of estrogen receptor (ER)-β but not of ER-α, protected against the development of aneurysmal rupture. **A,** Incidence of ruptured and unruptured aneurysms. **B,** Rupture rate. **C,** Survival curve. *vs vehicle.
In ovariectomized female mice (ie, menopausal mice), we found that the protective effect of ER-β activation was dependent on the production of nitric oxide. Similar to our findings, cardioprotective effects of estrogen can be mediated by ER-β in a nitric oxide–dependent manner.\textsuperscript{18,21} The activation of ER-β can stimulate not only the expression of both inducible and endothelial nitric oxide synthases but also the production of nitric oxide.\textsuperscript{17} Both inducible and endothelial nitric oxide synthase are involved in acute and chronic inflammation, processes that are emerging as integral parts of the pathophysiology of intracranial aneurysms.\textsuperscript{22} The S-nitrosylation of various proteins by nitric oxide can prevent the oxidative modification of cysteine residues,\textsuperscript{18,21} thereby potentially reducing the excessive tissue remodeling that can lead to aneurysm rupture.

There are several limitations of our study. In our experiment, it is not clear which type of nitric oxide synthase is responsible for the protective effect of ER-β or which proteins undergo S-nitrosylation. Future studies should use isoform-specific nitric oxide synthase inhibitors and isoform-specific nitric oxide synthase knockout mice.

Another major limitation of the study is that we used relatively young female mice (10- to 12-week old). Female mice become reproductive at ≈7 weeks, and menopause occurs at ≈12 to 14 months.\textsuperscript{23} The majority of studies that assessed roles of estrogen during the postmenopausal period used ovariectomy in 8- to 10-week-old mice.\textsuperscript{14,24} However, ovariectomy in relatively young, premenopausal mice may not completely simulate physiological menopause that normally occurs in much older female mice.\textsuperscript{25} Ovariectomy may result in an abrupt loss of ovarian hormones and may bypass the perimenopausal stage that can be characterized by a gradual loss and fluctuation of estrogen.\textsuperscript{25} Effects of ovariectomy and estrogen therapy may be different between the early stage and the late stage of reproductive age because of the age-related changes in various tissues and their response.\textsuperscript{25,26} Moreover, there seems to be significant differences in the expression levels, sensitivity, and response of ERs between early and late stage of menopause.\textsuperscript{27,28} Further studies using older female mice may be needed to confirm the protective effect of ER-β activation against the development of aneurysmal rupture in postmenopausal female mice.

Menopause causes a loss of estrogen and progesterone. Progesterone can improve outcomes after ischemic and traumatic brain injury in animals partly through the modulation of inflammation and oxidative stress.\textsuperscript{29} There seem to be interactions between estrogen and progesterone in neuroprotection.\textsuperscript{29} In addition, testosterone can augment inflammation by modulating oxidative stress or by activating proinflammatory cytokines.\textsuperscript{29} Vascular inflammation in the brain may be influenced by the balance among estrogen, testosterone, and progesterone.

In our study, we chose dosages of 17β-estradiol, DPN, and propyl pyrazole triol based on previous publications.\textsuperscript{9–15} Although we used the previously established dosages of these agents, we did not establish the dose-dependent effects of each agent in this study. Although DPN is highly selective for ER-β, it still possesses a weak agonistic effect on ER-α (ER-α versus ER-β, 1:170).\textsuperscript{30} Propyl pyrazole triol, a selective ER-α, also has a weak agonistic activity on ER-β (ER-α versus ER-β, 1000:1).\textsuperscript{30}

We used the uterine weight as a bioassay of ER stimulation to confirm the efficacy of ovariectomy and drug treatment. Uterus is an estrogen-sensitive organ of which weight can be augmented by ER stimulation. Uterine weight has been successfully used to verify the biological activity of estrogen in mice, and the uterine weight closely correlates with the plasma estrogen levels.\textsuperscript{9,11–15,24} However, the lack of the direct measurement of blood estrogen levels remains a limitation of this study.

**Perspectives**

In this study, we found the protective effect of estrogen, mainly through ER-β, against the development of aneurysmal rupture. Unwanted effects of estrogen, such as increased risks of breast cancer and endometrial cancer in certain populations, are often attributed to its agonistic activity without tissue specificity. To avoid the unwanted effects of estrogen, selective ER modulators that exert agonistic or antagonistic actions on ERs in a tissue-specific fashion are under vigorous investigation. Our findings may become the basis for testing selective ER modulators with a favorable tissue specificity profile to prevent the growth and rupture of intracranial aneurysms in humans, particularly in postmenopausal women.

**Sources of Funding**

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\*P<0.05 vs vehicle, †P<0.05 vs N-nitro-L-arginine methyl ester (L-NAME), and #P<0.05 vs ER-β+L-NAME.

Figure 5. The protective effect of estrogen receptor (ER)-β stimulation was dependent on nitric oxide production. A, Incidence of ruptured and unruptured aneurysms. B, Rupture rate. C, Survival curve.
References


Novelty and Significance

What Is New?

• Using a mouse model of intracranial aneurysm, we found that estrogen can protect against the development of intracranial aneurysm rupture in ovariec tomized mice through the activation of estrogen receptor-β.

What Is Relevant?

• Our data are relevant to the development of the new pharmacological treatment for the prevention of aneurysmal subarachnoid hemorrhage in postmenopausal women who are at the high risk for aneurysmal subarachnoid hemorrhage.

Summary

Estrogen receptor-β seems to play critical role in protective effects of estrogen against intracranial aneurysmal rupture in estrogen-deficient state.
Estrogen Protects Against Intracranial Aneurysm Rupture in Ovariectomized Mice
Yoshiteru Tada, Kosuke Wada, Kenji Shimada, Hiroshi Makino, Elena I. Liang, Shoko Murakami, Mari Kudo, Fumiaki Shikata, Ricardo A. Pena Silva, Keiko T. Kitazato, David M. Hasan, Yasuhisa Kanematsu, Shinji Nagahiro and Tomoki Hashimoto

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ESTROGEN PROTECTS AGAINST INTRACRANIAL ANEURYSM RUPTURE IN OVARIECTOMIZED MICE

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From Department of Anesthesia and Perioperative Care (YT, KW, KS, HM, EIL, SM, MK, FS, TH), University of California, San Francisco, CA; Department of Neurosurgery (YT, KTK, YK, SN), School of Medicine, The University of Tokushima, Tokushima City, Japan; Department of Neurosurgery, Carver College of Medicine, University of Iowa, Iowa City, IA (RAPS, DMH).

*Y.T. and K.W. contributed equally to the project.
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Methods

Experiments were conducted in accordance with the guidelines approved by the University of California, San Francisco, Institutional Animal Care and Use Committee. Intracranial aneurysms were induced in 10- to 12-week-old female mice (C57BL/6J, Jackson Laboratory, Bar Harbor, ME, USA) as previously described.\textsuperscript{1-3} We combined induced systemic hypertension and a single injection of elastase into the cerebrospinal fluid at the right basal cistern. To induce systemic hypertension, we used deoxycorticosterone acetate-salt hypertension (DOCA-salt hypertension).\textsuperscript{4}

To mimic menopause, 10- to 12-week-old female mice underwent bilateral ovariectomy.\textsuperscript{5} As part of the DOCA-salt hypertension, the mice underwent nephrectomy on the same day. One week later, a DOCA pellet (2.4 mg/day, Innovative Research of America, Sarasota, FL, USA) was implanted, and 1% sodium chloride drinking water was started.\textsuperscript{4} Mice received a single injection of elastase (0.035 units) into the cerebrospinal fluid at the right basal cistern on the same day as DOCA pellet implantation.\textsuperscript{3}

To detect aneurysmal rupture, two blinded observers performed daily neurological examination as previously described.\textsuperscript{3} Neurological symptoms were scored as follows: 0: normal function; 1: reduced eating or drinking activity demonstrated by a weight loss greater than two grams of body weight (approximately 10% weight loss) over 24 hours; 2: flexion of the torso and forelimbs upon lifting the whole animal by the tail; 3: circling to one side with a normal posture at rest; 4: leaning to one side at rest; and 5: no spontaneous activity. In our previous study, we have shown that this neurological testing system is sensitive and specific for detecting aneurysmal rupture in this model.\textsuperscript{3} Mice were euthanized when they developed neurological symptoms (score 1-5). All asymptomatic mice were euthanized 21 days after aneurysm induction. The brain samples were perfused with phosphate-buffered saline, followed by a gelatin containing blue dye to visualize cerebral arteries.

Two blinded observers assessed aneurysm formation and subarachnoid hemorrhage. Aneurysms were defined as a localized outward bulging of the vascular wall, whose diameter was greater than the parent artery diameter.\textsuperscript{1, 2} The rupture rate was defined as the total number of mice with ruptured aneurysms divided by the number of mice with any aneurysms.\textsuperscript{3}

Our previous study found that aneurysm formation occurs during the first 6 days after aneurysm induction in this model and that aneurysmal rupture begins to occur approximately 7 days after the aneurysm induction.\textsuperscript{3} By treating the mice with an experimental agent starting from 6 days after aneurysm induction, we were able to test whether the experimental agent can reduce the rupture rate without affecting the formation of aneurysms.\textsuperscript{3} Therefore, in this study, the treatments with estrogen (17β-estradiol), non-selective estrogen receptor antagonist (ER antagonist: ICI-182780: 3 mg/kg/day),\textsuperscript{6} propyl pyrazole triol (estrogen receptor-α agonist: PPT), or diarylpropionitrile (estrogen receptor-β agonist: DPN) were started 6 days after aneurysm induction so that the treatments affected the development of aneurysmal rupture without affecting aneurysmal formation. We used a 60-day release pellet that contained a vehicle; estrogen (17β-estradiol, 0.025 mg) (0.0004 mg/day); PPT, 2.5 mg (0.04 mg/day); or DPN, 2.5 mg (0.04 mg/day).\textsuperscript{7} Some of these mice received a nitric oxide synthase inhibitor, N-nitro-L-arginine methyl ester (L-NAME, 20 mg/kg/day), in drinking water starting 6 days after aneurysm induction.
Human intracranial aneurysm and superficial temporal artery tissues were collected according to the protocol approved by the University of Iowa Institutional Review Board (IRB). Two superficial temporal artery tissues and five aneurysm tissues were collected. Tissues were embedded in paraffin and stained with anti-estrogen receptor-α antibody (M7047 1D5, Dako, Carpinteria, CA, USA) or anti-estrogen receptor-β antibody (Ab3577, Abcam, Cambridge, MA, USA). Two observers who were blinded to the clinical information counted estrogen receptor-α and estrogen-β positive cells in randomly chosen areas. Semi-quantitative analysis of the slides was performed based on the immunostained positive cell counts per high-power field (40X): grade 0 = 0-10 cells; grade 1 = 10-20 cells; grade 2 = 20-30 cells; grade 3 = greater than 30 cells.
References


Supplementary table S1. Clinical characteristics and staining grade

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STA: superficial temporal artery; ICA: internal carotid artery; MCA: middle cerebral artery; ACOM: anterior communicating artery; ER: estrogen receptor

* from the same patient
Supplementary table S2. Systolic blood pressure and uterine weight

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ER: estrogen receptor; OVX: bilateral ovariectomy; L-NAME: N-nitro-L-arginine methyl ester