Published in final edited form as:
Hypertension. 2014 June ; 63(6): 1339–1344. doi:10.1161/HYPERTENSIONAHA.114.03300.

ESTROGEN PROTECTS AGAINST INTRACRANIAL ANEURYSM RUPTURE IN OVARIECTOMIZED MICE

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Abstract

Clinical observations suggest that post-menopausal women have a higher incidence of aneurysmal rupture than premenopausal women. We hypothesize that a relative deficiency in estrogen may increase the risks for aneurysmal growth and subarachnoid hemorrhage in post-menopausal women. We assessed the effects of estrogen and selective estrogen receptor subtype agonists on the development of aneurysmal rupture in ovariectomized female mice. We utilized an intracranial aneurysm mouse model that recapitulates the key features of human intracranial aneurysms, including spontaneous rupture. Ten- to twelve-week-old ovariectomized female mice received treatment with estrogen, non-selective 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. Non-selective estrogen receptor antagonist abolished the protective effect of estrogen. Though 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 appeared to occur through the activation of estrogen receptor-β, a predominant subtype of estrogen receptor in human intracranial aneurysms and cerebral arteries.

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Conflict of interest/Disclosure
None

Keywords

Intracranial aneurysm rupture; estrogen; menopause; animal model

Introduction

Clinical observations suggest that post-menopausal women have a higher incidence of aneurysmal subarachnoid hemorrhage than pre-menopausal women.[1] In addition, hormone replacement regimens that contain estrogen appear to reduce the risk for subarachnoid hemorrhage in post-menopausal women.[2] These epidemiological observations suggest the potentially protective role of estrogen against the development of aneurysmal rupture in post-menopausal women.[1, 3] Experimental studies using a rat model of intracranial aneurysms indicate the protective effect of estrogen against the formation of aneurysms.[4, 5] 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 subtype agonists on the development of aneurysmal rupture in ovariectomized female mice. Ovariectomized female mice were used to mimic the conditions of post-menopausal 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 post-menopausal women. We utilized an intracranial aneurysm mouse model that recapitulates the key features of human intracranial aneurysms, including spontaneous rupture.[6–8]

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.[6–8]. Bilateral ovariectomy or sham ovariectomy was performed one week prior to aneurysm induction. Detailed methods are presented in Online Data Supplements.

To detect aneurysmal rupture, two blinded observers performed daily neurological examination as previously described.[7] 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. 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.[6, 8] Figures 1A–1C show 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.

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.[7] Therefore, in this study, the treatments with estrogen (17β-estradiol, 0.17/mg/kg/day), non-selective estrogen receptor antagonist (ER antagonist: ICI-182780: 3 mg/kg/day),[9] propyl pyrazole triol (estrogen receptor-α agonist: PPT, 0.17/mg/kg/day), or diarylpropionitrile (estrogen receptor-β agonist: DPN, 0.17/mg/kg/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.[10–15]

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 one superficial temporal artery tissues were collected and stained with anti-estrogen receptor-α antibody (1D5, Dako, Carpinteria, CA, USA) or anti-estrogen receptor-β antibody (Ab3577, Abcam, Cambridge, MA, USA). Two blinded observers counted estrogen receptor 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.

Statistical Analysis

We used Fisher's 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 of the results were expressed as the mean ± SD. $P < 0.05$ was considered statistically significant.

Results

Expression of estrogen receptors in human intracranial aneurysms

We collected three human intracranial aneurysm tissues and a control artery (superficial temporal artery) from female patients underwent aneurysms clipping. Positive control tissues exhibited strong expression of both estrogen receptor-α and estrogen receptor-β (Figure 2). In the control artery, estrogen receptor-β was expressed in the smooth muscle layers, but estrogen receptor-α was almost absent (Figure 2). Similarly, there was no significant expression of estrogen receptor-α in the aneurysmal wall. Estrogen receptor-β was expressed in the smooth muscle layers and organized thrombus of the intracranial aneurysms (Figure 2). Supplementary table S1 shows the clinical characteristics and the semi-quantitative grading of estrogen receptor-α and estrogen receptor-β positive cells.

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 (i.e., post-menopausal mice), we treated ovariectomized mice with estrogen (17β estradiol), vehicle, or estrogen + non-selective estrogen receptor antagonist (ICI-182780) starting at 6 days after aneurysm induction. The treatments were continued for 2 weeks.

Treatments with estrogen or estrogen + non-selective estrogen receptor 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 (i.e., the incidence of both ruptured and unruptured aneurysms) among the three groups (Figure 3A). However, compared to the vehicle treatment, the estrogen treatment significantly reduced both the incidence of ruptured aneurysms (Figure 3A; vehicle control vs. estrogen 63% vs. 18%, $P < 0.01$) and the rupture rate (Figure 3B; vehicle control vs. estrogen 86% vs. 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 treatment with non-selective estrogen receptor antagonist (Figures 3A and 3B; incidence of intracranial aneurysms: estrogen vs. estrogen + estrogen receptor antagonist 18% vs. 59%, $P < 0.05$; rupture rate: estrogen vs. estrogen + estrogen receptor antagonist 23% vs. 71%, $P < 0.05$), confirming that the protective effect of estrogen was mediated by the activation of estrogen receptors.

Stimulation of estrogen receptor-β, but not of estrogen receptor-α, protected against the development of aneurysmal rupture

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

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

The protective effect of estrogen receptor-β stimulation depends on nitric oxide production

Estrogen can exert various effects on vasculature by increasing the production and availability of nitric oxide.[16] The stimulation of estrogen receptor-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.[17, 18] Therefore, we tested whether the protective effect of estrogen receptor-β stimulation against the development of aneurysmal rupture was dependent on the production of nitric oxide.

We treated mice with a non-specific 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 estrogen receptor-β agonist (DPN) (Figures 5A and 5B; incidence of ruptured aneurysms: L-NAME + DPN vs. DPN alone = 69% vs. 13%, $P < 0.01$; rupture rate: L-NAME + DPN vs. DPN alone = 79% vs. 18%, respectively, $P < 0.01$), indicating that the protective effect of estrogen receptor-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 DOCA-salt treatment. DOCA-salt induced hypertension probably masked the blood pressure augmentation effect of L-NAMA that is normally observed in normotensive animals. Similarly, estrogen or estrogen receptor agonist did not affect the blood pressure. Again, this is probably due to the hypertensive effect of DOCA-salt treatment that negated the effects from estrogen or estrogen receptor agonist (Supplementary Table S2).

Discussion

In this study, we showed that estrogen prevented aneurysmal rupture in ovariectomized female mice, consistent with the epidemiological studies.[1, 2] The protective effect of estrogen appeared to occur through the activation of estrogen receptor-β, a predominant subtype of estrogen receptor in human intracranial aneurysms and cerebral arteries. Furthermore, the protective effect of estrogen receptor-β stimulation was dependent on the production of nitric oxide. These findings support the causal and mechanistic link between the stimulation of estrogen receptor-β by estrogen and the prevention of aneurysmal rupture.

Estrogen receptor-α and estrogen receptor-β regulate different sets of genes and mediate different cellular and tissue effects.[19] For example, in vascular smooth muscle cells, estrogen receptor-β mediates the upregulation of nitric oxide synthase, but estrogen receptor-α exerts the opposite effect.[20] We found that estrogen receptor-β is a predominant form of estrogen receptor 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 estrogen receptor-β.

In ovariectomized female mice (i.e., menopausal mice), we found that the protective effect of estrogen receptor-β activation was dependent on the production of nitric oxide. Similar to

our findings, estrogen's cardioprotective effects can be mediated by estrogen receptor-β in a nitric oxide-dependent manner.[18, 21] The activation of estrogen receptor-β can stimulate not only the expression of both inducible and endothelial nitric oxide synthases but also the production of nitric oxide.[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.[22] The s-nitrosylation of various proteins by nitric oxide can prevent the oxidative modification of cysteine residues,[18, 21] thereby potentially reducing the excessive tissue remodeling that can leads to aneurysm rupture.

There are a number of limitations of our study. In our experiment, it is not clear which type of nitric oxide synthase is responsible for the protective effect of estrogen receptor-β or which proteins undergo s-nitrosylation. Future studies should utilize 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 (8–10 weeks-old). Female mice become reproductive approximately at 7 weeks, and menopause occurs at approximately 12–14 months.[23] The majority of studies that assessed roles of estrogen during the post-menopausal period used ovariectomy in 8–10 week-old mice.[14, 24] However, ovariectomy in relatively young, pre-menopausal mice may not completely simulate physiological menopause that normally occurs in much older female mice.[25] Ovariectomy may result in an abrupt loss of ovarian hormones and bypass the peri-menopausal stage that can be characterized by a gradual loss and fluctuation of estrogen.[25] Effects of ovariectomy and estrogen therapy may be different between the early stage and late stage of reproductive age, because of the age-related changes in various tissues and their response.[25, 26] Moreover, there appears to be significant differences in the expression levels, sensitivity, and response of estrogen receptors between early and late stage of menopause[27, 28] Further studies using older female mice may be needed to confirm the protective effect of estrogen receptor-β activation against the development of aneurysmal rupture in post-menopausal 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.[29] There seem to be interactions between estrogen and progesterone in neuroprotection.[29] In addition, testosterone can augment inflammation by modulating oxidative stress or activating pro-inflammatory cytokines.[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 PPT based on previous publications.[9–15] While 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 estrogen receptor-β, it still possesses a weak agonistic effect on estrogen receptor-α (estrogen receptor-α vs. estrogen receptor-β = 1:170).[30] PPT, a selective estrogen receptor-α, also has a weak agonistic activity on estrogen receptor-β (estrogen receptor-α vs. estrogen receptor-β = 1000:1).[30]

We used the uterine weight as a bioassay of estrogen receptor stimulation to confirm the efficacy of ovariectomy and drug treatment. Uterus is an estrogen sensitive organ of which weight can be augmented by estrogen receptor 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.[9, 13–15, 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 estrogen receptor-β, against the development of aneurysmal rupture. Estrogen's unwanted effects, such as increased risks for 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 estrogen receptor modulators (SERMs) that exert agonistic or antagonistic actions on estrogen receptors in a tissue specific fashion are under vigorous investigation. Our findings may become the basis for testing SERMs with a favorable tissue specificity profile to prevent the growth and rupture of intracranial aneurysms in humans, particularly in post-menopausal women.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding

The project described was supported by Grant Number R01NS055876 (TH), R01NS082280 (TH), and K08NS082363 (DMH) from the National Institute of Neurological Disorders and Stroke (NIH/NINDS), and the Brain Aneurysm Foundation Shirley Dudek Demmer Chair of Research (KS).

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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 ovariectomized mice through the activation of estrogen receptor-β.

What Is Relevant

Our data is relevant to the development of the new pharmacological treatment for the prevention of aneurysmal subarachnoid hemorrhage in post-menopausal women who are at the high risk for aneurysmal subarachnoid hemorrhage.

Summary

Estrogen receptor-β appears to play critical role in protective effects of estrogen against intracranial aneurysmal rupture in estrogen deficient state.

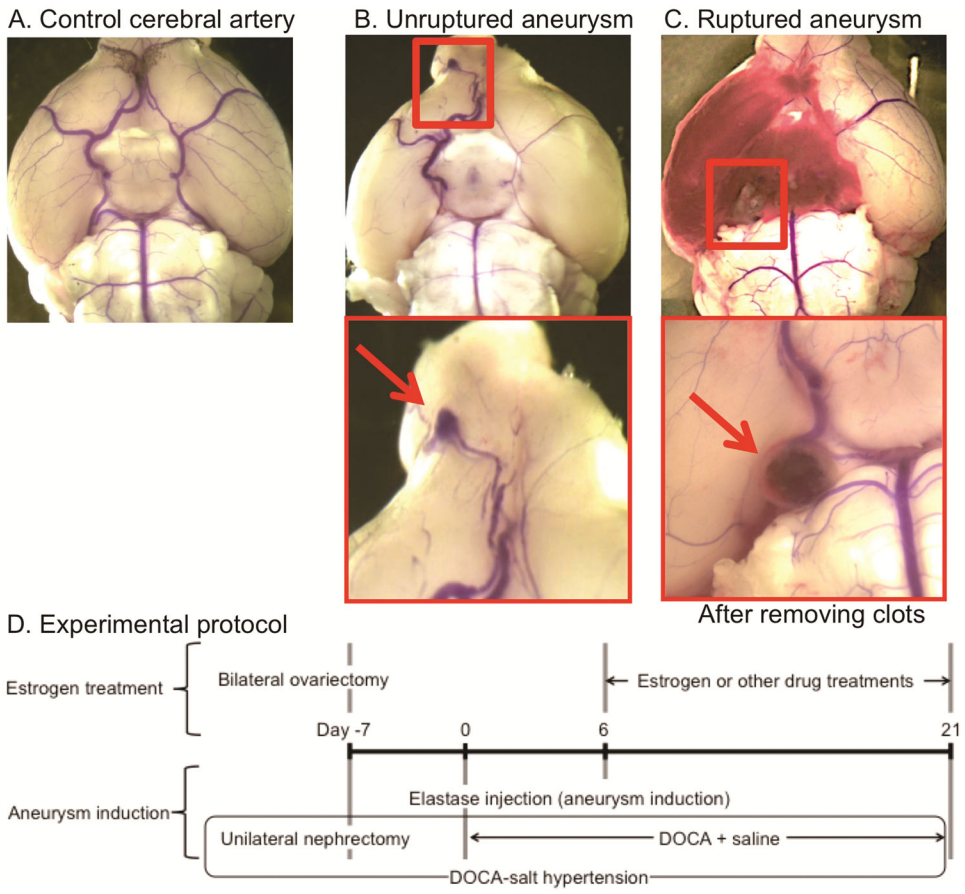

Figure 1.

A–C. Representative intracranial aneurysms in mice. A: Normal cerebral artery. B: Unruptured aneurysm in the anterior cerebral artery. C: Ruptured aneurysm with subarachnoid hemorrhage. **D. Experimental protocol to study the protective role of estrogen against the development of aneurysmal rupture.** DOCA: deoxycorticosterone acetate

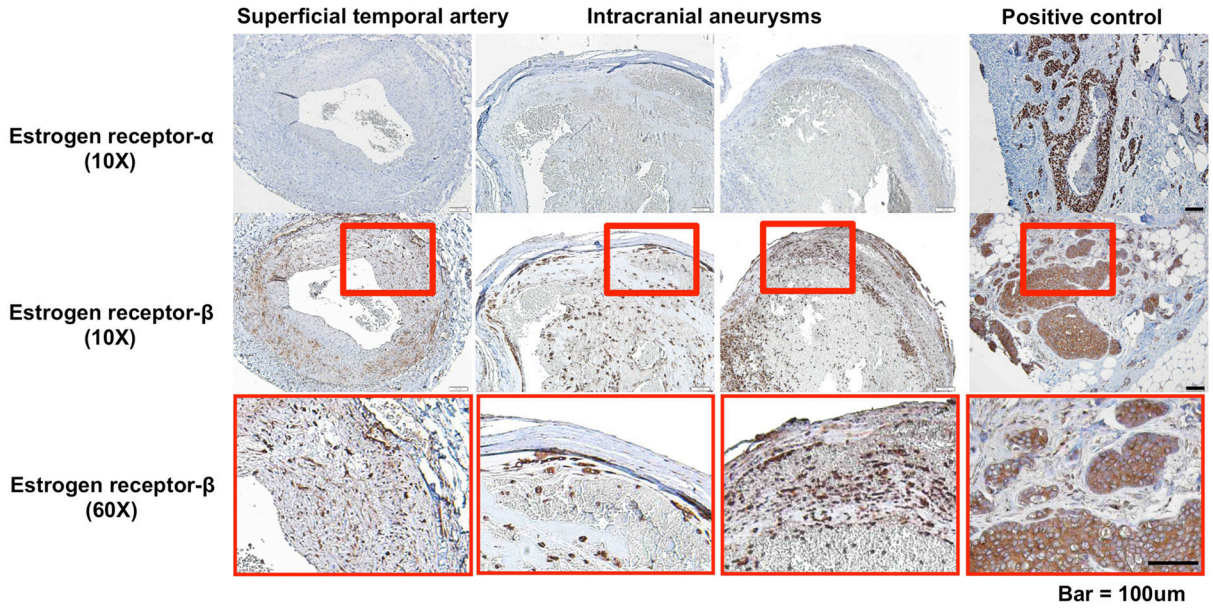

Figure 2. Estrogen receptors in intracranial aneurysms and control arteries in humans

Though estrogen receptor-α was almost absent in both human intracranial aneurysms and superficial temporal arteries, estrogen receptor-β was highly expressed in smooth muscle cell layers of the human intracranial aneurysms and superficial temporal arteries.

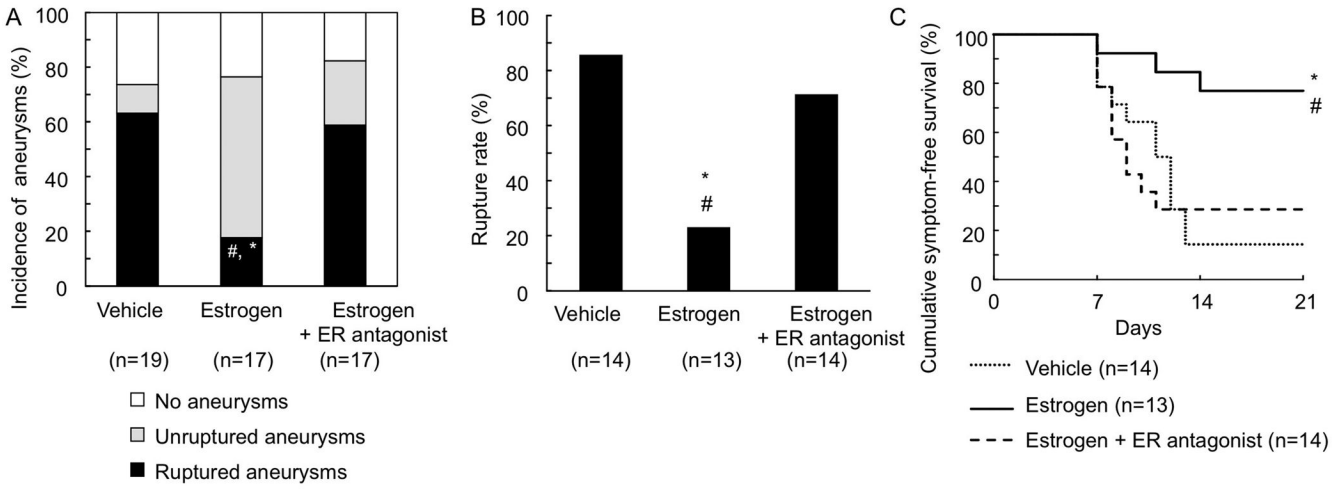

Figure 3. Estrogen protected against the development of aneurysmal rupture in ovariectomized female mice

A: Incidence of ruptured and unruptured aneurysms. B: Rupture rate. C: Survival curve. Survival curves were constructed after excluding those mice that did not have aneurysms so that the curves mimic clinical settings. * vs vehicle, # vs Estrogen + ER antagonist. ER: estrogen receptor.

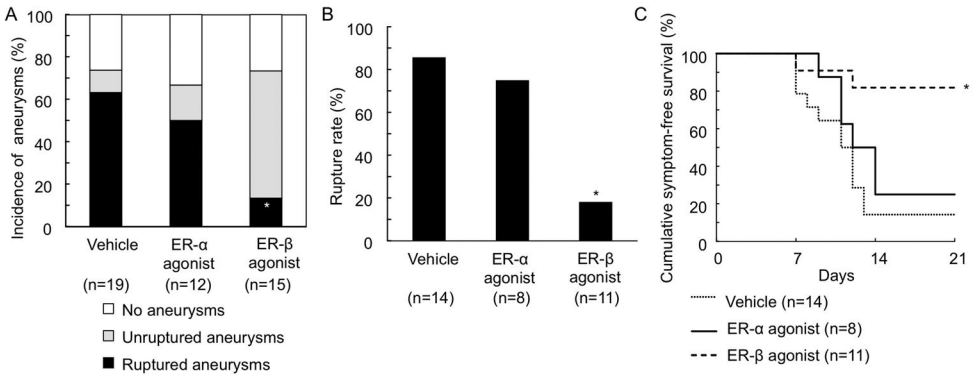

Figure 4. Stimulation of estrogen receptor-β, but not of estrogen receptor-α, protected against the development of aneurysmal rupture

A: Incidence of ruptured and unruptured aneurysms. B: Rupture rate. C: Survival curve. *vs vehicle

ER: estrogen receptor

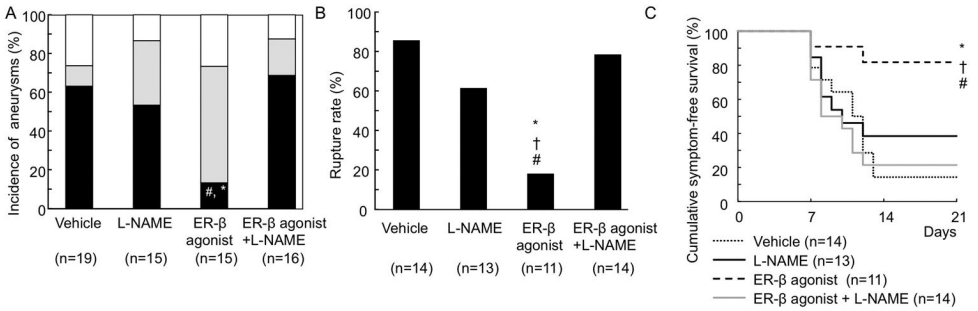

Figure 5. The protective effect of estrogen receptor-β stimulation was dependent on nitric oxide production

A: Incidence of ruptured and unruptured aneurysms. B: Rupture rate. C:Survival curve. * p vs vehicle, † vs L-NAME, # vs ER-β+L-NAME.