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Roles of estrogen in the formation of intracranial aneurysms in ovariectomized female mice

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Abstract

Background—Epidemiological studies have indicated that post-menopausal women have a higher incidence of intracranial aneurysms than men in the same age group.

Objective—We sought to investigate whether estrogen or estrogen receptors (ERs) mediate protective effects against the formation of intracranial aneurysms.

Methods—Intracranial aneurysms were induced in mice by combining a single injection of elastase into the cerebrospinal fluid with deoxycorticosterone acetate salt hypertension. The mice were treated with estrogen (17 β -estradiol), ER α agonist (propylpyrazole-triol), and ER β agonist (diarylpropionitrile) with and without a nitric oxide synthase inhibitor.

Results—The ovariectomized female mice had a significantly higher incidence of aneurysms than the male mice, which was consistent with past epidemiological studies. In ovariectomized female mice, an ER β agonist, but not an ER α agonist or 17 β -estradiol, significantly reduced the incidence of aneurysms. The protective effect of the ER β agonist was absent in the ovariectomized ER β knockout mice. The protective effect of the ER β agonist was negated by treatment with a nitric oxide synthase inhibitor.

Conclusions—The effects of gender, menopause, and estrogen treatment observed in this animal study were consistent with previous epidemiological findings. Stimulation of estrogen

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receptor- β was protective against the formation of intracranial aneurysms in ovariectomized female mice.

Keywords

Stroke [50] cerebral aneurysm; AVM, and subarachnoid hemorrhage; Stroke treatment – surgical [79] Aneurysm; AVM, hematoma; Stroke treatment – medical [74] other stroke treatment/medical; Vascular biology [97] other vascular biology

Introduction

Women are known to have a higher incidence of intracranial aneurysms than men. However, epidemiological studies show that the female preponderance of intracranial aneurysms becomes significant only after the fourth or fifth decades of life, during the peri- and post-menopausal periods.^{1, 2} Before the fourth or fifth decades, there is no difference between men and women in the incidence of intracranial aneurysms and subarachnoid hemorrhage.^{1, 2} These epidemiological observations suggest the potential roles of sex steroids, particularly estrogen, in the pathophysiology of intracranial aneurysms. In post-menopausal women, a relative deficiency in estrogen may increase the risks for aneurysmal formation and growth.^{1–5}

Previous studies have demonstrated the protective effects of estrogen against various types of vascular injury, particularly atherosclerosis.⁶ Estrogen exerts protective effects against vascular injury by modulating inflammation, nitric oxide production, cytokine and growth factor expression, and the reduction of oxidative stress.⁷ Effects of estrogen are primarily mediated by two nuclear hormone receptors (ligand-activated transcriptional factors): estrogen receptor- α and estrogen receptor- β . Both estrogen receptor- α and estrogen receptor- β are expressed in vascular cells, including endothelial cells and smooth muscle cells.

In this study, we assessed the effects of estrogen and selective estrogen receptor subtype agonists on the formation of intracranial aneurysms in female mice. We sought to investigate the receptor subtype and the underlying mechanisms that are responsible for the potentially protective effect of estrogen.

Materials and Methods

Animal model

Experiments were conducted in accordance with the guidelines approved by the University of California, San Francisco's Institutional Animal Care and Use Committee. We used 10- to 12-week-old C57BL/6J mice and estrogen receptor- β knockout (ER β KO) mice (Jackson Laboratory, Bar Harbor, Maine). Intracranial aneurysms were induced by a combination of pharmacologically-induced hypertension and a single injection of elastase (10 milli-units) into the cerebrospinal fluid at the right basal cistern as we previously described.^{8–11} See Text, Supplemental Content 1, which demonstrates details of the aneurysm model. Four weeks after the aneurysm induction, we euthanized the mice and perfused the animals with bromophenol blue dye. Two blinded observers assessed the formation of intracranial

aneurysms by examining the Circle of Willis and its major branches under a dissecting microscope (10X). Intracranial aneurysms were operationally defined as a localized outward bulging of the vascular wall in the Circle of Willis or in its major primary branches, as previously described.¹¹

Treatment with estrogen, estrogen receptor- α agonist, and estrogen receptor- β agonist

In female mice, bilateral ovariectomy or sham ovariectomy was performed one week before the aneurysm induction (elastase injection). Immediately after the bilateral ovariectomy, we implanted a 60-day release pellet (Innovative Research of America, Sarasota, FL) that contained a vehicle, estrogen (17 β -estradiol, 0.025 mg), propyl pyrazole triol (estrogen receptor- α agonist) (PPT, 2.5 mg), or diarylpropionitrile (estrogen receptor- β agonist) (DPN, 2.5 mg). Some of these mice received an inhibitor of the nitric oxide synthase inhibitor, N-nitro-L-arginine methyl ester (L-NAME, 20 mg/kg/day) in drinking water.

Statistical analysis

All of the results were expressed as mean $\pm$ SD. Fisher's exact test was used to analyze the incidence of aneurysms. Differences between multiple groups were analyzed by a one-way ANOVA, followed by the Tukey-Kramer post hoc test. Statistical significance was accepted at $P < .05$. GraphPad Prism 6 was used for statistics software.

Results

Incidence of aneurysms in male mice, female mice, ovariectomized female mice, and ovariectomized female mice with estrogen treatment

As a first step to assess the effects of gender, menopause, and estrogen on the formation of aneurysms, we compared the incidence of aneurysms in (1) male mice with sham ovariectomy (laparotomy), (2) female mice with sham ovariectomy, (3) female mice with bilateral ovariectomy (surgical menopause), and (4) ovariectomized female mice with estrogen treatment (surgical menopause + estrogen replacement).

Figure 1 shows representative intracranial aneurysms that were observed in ovariectomized female mice. As shown in Figure 2, there was a trend for the bilateral ovariectomy—surgical menopause—to increase the incidence of aneurysms in female mice (59% vs. 36%, $P = .05$). More importantly, the ovariectomized female mice had a significantly higher incidence of aneurysms than the male mice with sham ovariectomy (59% vs. 15%, $P < .01$), consistent with the epidemiological studies that showed the female preponderance of aneurysms after peri-menopausal age.^{1, 2} There was also a trend for the estrogen treatment to reduce the incidence of aneurysms in ovariectomized female mice (38% vs. 59%, $P = .06$).

Table in the Supplemental Content 2 demonstrates systolic blood pressure and uterine weight of each group.

Stimulation of estrogen receptor- β but not stimulation of estrogen receptor- α protects against aneurysm formation

To identify the estrogen receptor subtype that was responsible for the protective role of estrogen, we treated the ovariectomized female mice with estrogen receptor- α agonist (PPT) or estrogen receptor- β agonist (DPN). Although the treatment with estrogen receptor- α agonist did not affect the incidence of aneurysms in the ovariectomized mice, the treatment with estrogen receptor- β agonist significantly reduced the incidence of aneurysms in ovariectomized mice (26% vs. 59%, $P < .05$) (Figure 3).

To further confirm the protective role of DPN is through estrogen receptor- β stimulation, ovariectomized estrogen receptor- β knockout mice were treated with DPN or vehicle. The protective effect of DPN treatment was abolished in estrogen receptor- β knockout mice (50% vs. 57%) (Figure 4), indicating that the protective effect of DPN was primarily attributed to its activation of estrogen receptor- β .

We further assessed the role of estrogen receptor- β by inducing intracranial aneurysms in estrogen receptor- β knockout mice with sham ovariectomy. There was a trend for the sham-ovariectomized estrogen receptor- β knockout mice to have higher incidence of aneurysms than wild-type mice with sham ovariectomy (67% vs. 36%, $P = 0.10$) (Figure 5).

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.¹² Stimulation of estrogen receptor- β can up-regulate nitric oxide production, at least partially, through the s-nitrosylation of various target proteins.^{13, 14} Therefore, we tested whether the protective effect of estrogen receptor- β stimulation against aneurysm formation 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). As shown in Figure 6, although L-NAME alone did not affect the incidence of aneurysms in ovariectomized mice, it abolished the protective effect of DPN (L-NAME + DPN vs. DPN alone = 65 % vs. 26%, respectively, $P < .05$), indicating that the protective effect of estrogen receptor- β stimulation against the formation of aneurysms depends on the production of nitric oxide by nitric synthase.

Discussion

In this study, we found that ovariectomized female mice had a higher incidence of aneurysms than male mice and that there was no difference in the incidence of aneurysms between non-ovariectomized female mice and male mice. These findings were generally consistent with human epidemiological studies revealing that the female preponderance of intracranial aneurysms becomes significant only after the fourth or fifth decades (in peri- and post-menopause).^{1, 2} The consistencies between the animal data and human epidemiological observations support the rationale for using this animal model to study potential mechanisms of the gender difference in the pathophysiology of intracranial aneurysms.

The estrogen receptor- β agonist significantly reduced the incidence of aneurysms in ovariectomized mice; however, the estrogen receptor- α agonist failed to reduce the incidence of aneurysms. The protective effect of estrogen receptor- β stimulation was further supported by the lack of protective effect of estrogen receptor- β agonist in mice that lacked estrogen receptor- β . Estrogen receptor- α and estrogen receptor- β regulate different sets of genes and mediate different cellular and tissue effects.¹⁵ For example, in vascular smooth muscle cells, while estrogen receptor- β mediates the upregulation of an inducible form of nitric oxide synthase, estrogen receptor- α exerts the opposite effect.¹⁶ In cardiomyocytes, inducible nitric oxide synthase is involved in both acute and chronic inflammation; these processes are emerging as an integral part of the pathophysiology of intracranial aneurysms.^{17, 18} In our experiment, we found that the protective effect of estrogen receptor- β activation was dependent upon the production of nitric oxide. Similar to our findings, it has been reported that estrogen's cardioprotective effects are mainly mediated by estrogen receptor- β in a nitric oxide-dependent manner.^{14, 19} Nitric oxide synthase is involved in acute and chronic inflammation, processes that are emerging as an integral part of the pathophysiology of intracranial aneurysms.^{17, 18, 20} The s-nitrosylation of various proteins by nitric oxide can prevent the oxidative modification of cysteine residues,^{14, 19} thereby potentially reducing the excessive tissue remodeling that leads to aneurysm formation.¹⁷

It should be noted that there are a number of factors that can potentially limit the translational potential of our findings. First, the animal models do not completely replicate biological events that lead to aneurysm formation and growth. Second, the surgical menopause may not faithfully mimic the hormonal changes that occur in women at the time of age-related menopause. Third, there may be differences in the effects of estrogen or estrogen- β agonist between humans and mice. Fourth, we have not completely identified the exact process that can be modulated by the estrogen receptor- β agonist.

Our findings were generally consistent with previous experimental studies that utilized a rat model of intracranial aneurysm.^{21–23} While aneurysms in our mouse model were induced by a combination of DOCA-salt hypertension and a single injection of elastase, the previous studies that used rats utilized a combination of unilateral carotid artery ligation, renal hypertension, and ovariectomy. The fact that these two different models yielded similar findings on the role of estrogen in the formation of aneurysms may indicate that these two models share similar or identical downstream processes that lead to aneurysm formation. Utilizing the mouse model, we were able to establish the role of estrogen receptor- β in the protection against the formation of aneurysms.

The female mice reach reproductive maturity at approximately 7 weeks, and menopause occurs at approximately 12–14 months. We used mice aged 8–12 weeks, an age range that has been used by the majority of studies that assessed roles of estrogen during the post-menopausal period.^{24, 25} Ovariectomy in pre-menopausal mice may not completely simulate physiological menopause.²⁶ Ovariectomy may result in the abrupt loss of ovarian hormones and bypass the peri-menopausal stage, which can be characterized by a gradual loss and fluctuation of estrogen. Further studies using older female mice may be needed to confirm the protective effect of estrogen receptor- β activation against aneurysm formation in post-menopausal female mice.

Menopause causes a relative loss of estrogen and progesterone. Progesterone can improve outcomes after ischemic and traumatic brain injury in animals partly through modulation of inflammation and oxidative stress.²⁷ There seem to be interactions between estrogen and progesterone in neuroprotection.²⁷ In addition, testosterone can augment inflammation by modulating oxidative stress or activating pro-inflammatory cytokines.²⁸ Vascular inflammation in the brain may be influenced by the balance among estrogen, testosterone, and progesterone.²⁸ Thus, other sex steroids and their interactions should be studied in future studies. The gender difference in the incidence of aneurysms may be determined by the complex interactions and balance of sex steroids.

One of the limitations of this study is that we did not directly measure the blood estrogen levels. Instead, we measured the uterine weight to assess the stimulation of estrogen receptor, primarily estrogen receptor- α , as previously described.^{14, 29} 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).²⁶ 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 Estrogen and PPT, but not DPN, significantly increased the uterine weight in the ovariectomized mice in a manner consistent with previous studies,^{14, 31} which indicated the specificity of DPN and the efficacy of estrogen and PPT in our experiments.

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.³² To avoid the unwanted effects of estrogen, selective estrogen receptor modulators (SERMs) with tissue specificity are under vigorous investigation. SERMs are synthetic compounds that act as estrogen receptor agonists in some tissues and estrogen receptor antagonists in other tissues.³³ Our finding of the superior protective effect of the selective estrogen receptor- β agonist compared to estrogen may become the basis for testing SERMs with a favorable tissue specificity profile for preventing the growth and rupture of intracranial aneurysms in humans, particularly in post-menopausal women.

Conclusion

The effects of gender, menopause, and estrogen treatment observed in this animal study were consistent with previous epidemiological findings. Stimulation of estrogen receptor- β may confer the protective effect against the formation of aneurysms in post-menopausal women.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Representative intracranial aneurysms (arrows) in ovariectomized mice
Aneurysm formation at the olfactory artery (A) and superior cerebral artery (B). Blood vessels were perfused with bromophenol blue. Bar=1 mm. OA: olfactory artery; ACA: anterior cerebral artery; SCA: superior cerebral artery; BA: basilar artery.

Figure 2. Incidence of intracranial aneurysms in male and female mice

The incidence of aneurysms in female mice tended to be greater than in male mice (36 vs. 15%, $P = .17$). In addition, there was a strong trend for the bilateral ovariectomy (surgical menopause) to increase the incidence of aneurysms in female mice (59 vs. 36%, $P = .05$). Ovariectomized female mice had a significantly higher incidence of aneurysms than male mice (59 vs. 15%, $P < .01$). There was also a trend for the estrogen treatment to reduce the incidence of aneurysms in ovariectomized female mice (38 vs. 59%, $P = .06$)

OVX: bilateral ovariectomy. E2: estrogen treatment with 17β -estradiol.

Figure 3. Effects of estrogen receptor- α agonist and estrogen receptor- β agonist on the incidence of aneurysms in ovariectomized mice

The treatment with estrogen receptor- α agonist did not affect the incidence of aneurysms in ovariectomized mice; however, the treatment with estrogen receptor- β agonist significantly reduced the incidence of aneurysms in ovariectomized mice (26 vs. 59%, $P < .05$). ER: estrogen receptor

Figure 4. Effects of estrogen receptor- β agonist in ovariectomized estrogen receptor- β knockout mice

Unlike in wild-type mice, estrogen receptor- β agonist did not affect the incidence of aneurysms in ovariectomized estrogen receptor- β knockout mice (ER β KO). OVX: bilateral ovariectomy.

Figure 5.
Formation of aneurysms in sham ovariectomized estrogen receptor- β knockout mice and wild-type mice.

Figure 6. The protective effect of estrogen receptor- β agonist was abolished by the inhibition of nitric oxide synthase

A nitric oxide synthase inhibitor, N-nitro-L-arginine methyl ester (L-NAME), did not affect the incidence of aneurysms in ovariectomized mice. However, L-NAME abolished the protective effect of estrogen receptor- β agonist in ovariectomized mice (65 vs. 26%, $P < .05$). *: $P < .05$, **: $P < .01$ compared to OVX. #: $P < 0.05$ compared to OVX + ER β . OVX: bilateral ovariectomy. ER: estrogen receptor