The effect of 17β-estradiol in attenuating experimental subarachnoid hemorrhage–induced cerebral vasospasm

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Object. Sex differences in the outcome of aneurysmal subarachnoid hemorrhage (SAH) are controversial, and the potential influence of estradiol on vasodilation is unclear. In the present study the authors evaluate the effect and possible mechanism of 17β-estradiol (E2) on SAH-induced vasospasm in a two-hemorrhage rodent model of SAH.

Methods. A 30-mm Silastic tube filled with E2 in corn oil (0.3 mg/ml) was subcutaneously implanted in male rats. Serum levels of E2 were measured on Days 0, 1, 2, 3, 4, and 7 postimplantation. The degree of vasospasm was determined by averaging the cross-sectional areas of the basilar artery (BA) 7 days after the first SAH. Expressions of endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) in the BA were also evaluated.

Serum levels of E2 in the E2-treated rats were at physiological levels (56–92 pg/ml) and were significantly higher than those in the control and vehicle-treated groups. Treatment with E2 significantly (p < 0.01) attenuated SAH-induced vasospasm. Induction of iNOS messenger (m)RNA and protein in the BA by SAH was significantly diminished by the E2 treatment but not by vehicle treatment. The SAH-induced suppression of eNOS mRNA and protein was relieved by E2 treatment.

Conclusions. These results suggest that continuous treatment with E2 at physiological levels prevents cerebral vasospasm following SAH. The beneficial effect of E2 may be in part related to the prevention of augmentation of iNOS expression and the preservation of normal eNOS expression after SAH. Treatment with E2 holds therapeutic promise in the treatment of cerebral vasospasm following SAH and merits further investigation.

KEY WORDS • estrogen • subarachnoid hemorrhage • cerebral vasospasm • nitric oxide • rat

Although cerebral vasospasm after aneurysmal SAH has been recognized for more than half a century, it remains a major complication in patients suffering from SAH.4 Given that no effective treatment for cerebral vasospasm presently exists, the pathophysiological mechanism contributing to arterial dysfunction needs intensive study. Unlike other kinds of strokes, aneurysmal SAH occurs more frequently in women than in men.6 Differences between the sexes in outcomes of SAH are controversial, and the influence of the female sex hormone is unclear.7 Estrogens have been found to induce vasodilation in the cardiovascular system via both genomic and nongenomic mechanisms through generation of NO, cyclic guanine monophosphate, cyclic adenosine monophosphate, adenosine, and prostacycline, or by alterations in ion channel activity.5,8 The effect of estrogen on SAH-induced vasospasm, however, remains uncertain. Impaired endothelium-dependent relaxation in large cerebral arteries after experimental SAH has been demonstrated in different animal models,15,16,20 and in the BAs of patients after SAH.13 Altered production or activity of NO is probably due to a deficiency of eNOS in vasospastic cerebral vessels after SAH.15 Overproduction of NO under conditions of stress is associated with altered iNOS expression, which has been found in both endothelial and smooth muscle cells after SAH.37,41 Because pretreatment with aminoguanidine, a selective inhibitor of iNOS, ameliorated vascular constriction after SAH,35 we can infer from these results that iNOS and/or eNOS plays different roles in mediating vascular tone after SAH.

The role of estrogen in the cardiovascular system can be demonstrated in premenopausal women, who have a much lower cardiovascular risk than men. Estrogen replacement therapy significantly decreases the incidence of coronary heart disease in postmenopausal women.30 Estrogen has been shown to lower the incidence of mortality and reduce secondary ischemic damage after SAH in rats;41 however, the vascular effects of estrogen in SAH remain to be determined. It is widely accepted that NO accounts for most of the endothelium-dependent relaxation, and induction of

Abbreviations used in this paper: BA = basilar artery; eNOS = endothelial nitric oxide synthase; E2 = 17β-estradiol; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; iNOS = inducible NOS; NO = nitric oxide; mRNA = messenger RNA; PCR = polymerase chain reaction; SAH = subarachnoid hemorrhage; SEM = standard error of the mean.
eNOS by estrogen has been demonstrated in vascular endothelial cells through genomic and nongenomic mechanisms. Expression of iNOS following cytokine treatment is abrogated by estrogen treatment in vascular smooth muscle cells and implicates a beneficial role of estrogen under stress conditions. Whether both iNOS and eNOS participate in the effect of E2 in preventing vasospasm after SAH needs further evaluation. In the present paper we report findings on influence of E2, the most active natural estrogen, on SAH-induced vasospasm in an experimental rodent model of SAH. Furthermore, we show the influence of E2 on expression of eNOS and iNOS in the BA following SAH.

Materials and Methods

Animal Preparation

All procedures were approved by the Kaohsiung Medical University Animal Care and Use Committee. Ninety-six Sprague–Dawley male rats, each weighing between 350 and 420 g, were randomly divided evenly into four groups: 1) control (no SAH); 2) SAH only; 3) SAH plus treatment with vehicle; 4) SAH plus treatment with E2 (subcutaneous implantation of a 30-mm-long Silastic tube, which had a 2-mm inner diameter and a 4-mm outer diameter [Shin-Etsu Polymer Co., Ltd., Tokyo, Japan] and contained 0.3 mg/ml E2 benzoate in corn oil). The dosage of E2 was chosen based on findings of pilot studies in which this regimen produced physiological serum levels of estrogen (17–88 pg/ml).

An additional nine rats were divided into the following three groups to assess serum levels of E2: 1) control (no SAH); 2) E2 treatment; and 3) corn oil (vehicle) treatment. For the vehicle-treated group, a 30-mm-long Silastic tube containing corn oil only was implanted subcutaneously. For the confirmation of physiological levels of E2, blood from each rat was sampled through the tail artery just before implantation (Day 0) and at 1, 2, 3, 4, and 7 days after implantation. The Immulit 2000 chemiluminescence assay system (Diagnostic Products Corp., Los Angeles, CA) was utilized to determine serum E2 concentration.

Induction of Experimental SAH

The double-hemorrhage rodent model of SAH has been previously characterized. The rats were anesthetized by an intraperitoneal injection of pentobarbital (50 mg/kg). The animals' rectal temperature was controlled at 36 ± 1°C by using a heating pad (Harvard Apparatus, Holliston, MA), and the tail artery was cannulated with a polyethylene catheter for monitoring blood pressure and heart rate. The cisterna magna was punctured percutaneously with a 25-gauge butterfly needle. Approximately 0.1 to 0.15 ml of cerebrospinal fluid was slowly withdrawn and the junction between the needle and tube was clamped. Fresh autologous, nonheparinized blood (0.3 ml) was withdrawn from the tail artery. Using a needle-in-needle method (inserting a 30-gauge needle into the 25-gauge butterfly needle at the junction of the needle and tube), blood was injected slowly into the cisterna magna. The same procedure was repeated 48 hours later. Thus, in this study, the term SAH refers to an experimental model of SAH produced by a double injection of blood into the cisterna magna (two-hemorrhage SAH model). Seven days after the first SAH occurred, each rat was killed by a paraformaldehyde perfusion, and a portion of brain tissue was removed, placed in a fixative solution, and stored at 4°C overnight. Then, the BAs were isolated so that we could perform a tissue morphometric analysis. To study mRNA expression and the protein contents of iNOS or eNOS, the BAs were sampled after an intracardiac perfusion with normal saline.

Tissue Morphometric Analysis

The middle third of the BA was dissected for the morphometric analysis. Cross-sections (0.5 μm in thickness) of the BAs were cut, mounted on glass slides, and stained with 0.5% toluidine blue. Five randomly selected cross-sections of BAs from each animal were analyzed, and cross-sectional areas were measured using a computer-assisted morphometry system (Image 1; Universal Imaging Corp., Downingtown, PA) as described previously. For group comparisons an analysis of variance with the Bonferroni post hoc test was performed. Differences were considered to be significant at a probability level less than 0.05.

The Reverse Transcription–PCR Amplification of eNOS and iNOS mRNA

Three BAs were pooled for total RNA extraction and homogenized in 1 ml TRIzol reagent (GIBCO BRL, Grand Island, NY). The first complementary DNA strand was reverse transcribed from 5 μg total RNA. The forward and reverse PCR primers for eNOS were 5'-GGTGGA-CACAAGGCTGCGCA-3' and 5'-GAAGTAAGT-GAGAGCCTGGCG-CA-3', respectively. The forward and reverse PCR primers for iNOS were 5'-CCAAGACGTGTTCCAC-ATG-
3', and 5'-GAATGTCCAGGAGTAGGTGAGG-3', respectively. We used GAPDH as an internal control. The 5' primer 5'-TATGATGACATCAAGAAGTG-3' and the 3' primer 5'-CAC-CACCCTGTGG-CGTGTA-3' were used. The amplification profile involved denaturation at 94˚C for 1 minute, primer annealing at 60˚C (iNOS and GAPDH) or at 69˚C (eNOS) for 30 seconds, and extension at 72˚C for 1 minute and repeated 30 cycles. The PCR products of eNOS, iNOS, and GAPDH were 588, 317, and 219 bp, respectively, and were observed with the aid of ultraviolet light after a 2% agarose gel electrophoresis.

Western Blot Analysis of eNOS and iNOS

To each sample, nine volumes of dissecting buffer (50 mM Tris acetate, pH 7.4, 10% sucrose, 5 mM ethylenediamine tetraacetic acid) were added. After homogenization, the suspension was centrifuged at 16,000 G for 30 minutes, and the resulting pellets were resuspended, rehomogenized, and stored at −70˚C. The protein concentration was estimated using a protein microassay procedure (Bio-Rad Corp., Hercules, CA). Equal amounts of protein (20 µg for eNOS and 50 µg for iNOS) were separated using a 7.5% sodium dodecyl sulfate–polyacrylamide gel and were transferred onto a polyvinylidene difluoride membrane by electroblotting for 1 hour at 100 V. The membrane was incubated overnight at 4˚C with blocking buffer containing 5% nonfat dry milk. The following day the blot was incubated with either eNOS or iNOS antibody (polyclonal; Santa Cruz Laboratories, Santa Cruz, CA) at a 1:50 or 1:500 dilution, respectively, for 1 hour, and then incubated for 1 hour with goat anti-rabbit immunoglobulin G (HRP conjugated, Santa Cruz Laboratories) at a 1:3000 dilution. Immunoreactive protein was visualized by enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, United Kingdom) according to the manufacturer’s specifications.

Results

General Observations

Before the perfusion–fixation procedure, there were no significant differences among the control and treated groups of animals in any of the physiological parameters that were recorded, including body weight, mean arterial blood pressure, and heart rate (data not shown).

Serum Levels of E2

Implantation of an E2 Silastic tube produced stable elevations of serum E2 levels for at least 7 days. The physiological serum levels of E2 in the E2 treatment group (56–92 pg/ml) was significantly higher than those in the control group (26–40 pg/ml) and the SAH plus vehicle-treated group (28–36 pg/ml) (p < 0.01 at Day 1, 2, 3, 4; p < 0.05 at Day 7; Fig. 1).

Cross-Sectional Luminal Area Measurements

The cross-sectional area of the BAs was significantly reduced in animals subjected to SAH (p < 0.01; 12 per each group; Fig. 2B and C). When compared with animals in the control group (mean ± SEM 54,511 ± 3248 µm²; Fig. 2A), areas in the SAH only (28,436 ± 2386 µm²) and the SAH plus vehicle-treated group (28,080 ± 1115 µm²) groups were significantly reduced by 48 and 49%, respectively. No SAH-induced reduction in arterial area was found in animals treated with E2 (56,857 ± 2188 µm²; Fig. 2D). The cross-sectional areas in the E2 treatment group differed significantly from those in the SAH only and SAH plus vehicle treatment groups (p < 0.01; Fig. 3).

The Expression of eNOS and iNOS mRNA

For this study, values are expressed as means ± SEMs of three samples and each sample constitutes the pooling of...
three BAs obtained in three rats. The expression of eNOS mRNA in the SAH and the SAH plus vehicle treatment groups decreased significantly by 47 and 40%, respectively, when compared with the control group. No decrease in the expression of eNOS mRNA was observed following E2 treatment, however, when compared with the control group (Fig. 4A). The expression of iNOS mRNA was increased approximately threefold in animals subjected to SAH, when compared with control animals (p < 0.01); however, SAH-induced iNOS mRNA expression was significantly (p < 0.01) diminished by E2 treatment, but not by vehicle treatment (Fig. 5A).

**Protein Contents of eNOS and iNOS**

For this study, values are again expressed as means ± SEMs of three samples and each sample constitutes the pooling of three BAs obtained in three rats. The protein content of eNOS was significantly less in the SAH (p < 0.05) and SAH plus vehicle treatment (p < 0.01) groups than that in the control group. Nevertheless, a significant difference in the protein content of eNOS was observed between the E2 treatment group and the SAH only (p < 0.05) or SAH plus vehicle treatment (p < 0.01) groups (Fig. 4B). No significant difference in the eNOS protein content was observed between the control and the E2 treatment group.

The protein content of iNOS was significantly higher in the SAH only and SAH plus vehicle treatment group than that in the control group (p < 0.01). Nevertheless, the protein content of iNOS in the E2 treatment group was similar to that in the control group. There were significant differences between the E2 treatment group and the SAH only or SAH plus vehicle treatment groups (p < 0.01; Fig. 5B).

**Discussion**

Delayed cerebral vasospasm is a major cause of death and neurological morbidity in patients afflicted with aneurysmal SAH. Systemic administration of vasodilators may reverse cerebral vasospasm, although potential hypotensive effects may compromise global cerebral blood flow. Enhancement of endothelium-dependent vasodilatory function without significant systemic effects is an admirable goal to combat SAH-induced vasospasm. Physiological levels of estrogen are devoid of hypotensive effects, and its potential protective effects on the cardiovascular system are well known in premenopausal women and in postmenopausal women who receive sex hormone replacement therapy. In the present study, E2 treatment prevented the cerebral vasospasm after SAH without significant changes in blood pressure. Therefore, estrogen may be a plausible candidate for the prevention of delayed cerebral vasospasm after SAH.

In our study, serum levels of E2 in the E2 treatment group (56–92 pg/ml) were significantly higher than those in the control (26–40 pg/ml) and SAH plus vehicle treatment (28–36 pg/ml) groups. All these levels (26–92 pg/ml) were nearly within the physiological range (17–88 pg/ml). In our
reported that E2 can reduce secondary ischemic damage and death due to SAH. Inhibition of iNOS has been shown to decrease cerebral vasospasm after SAH in rats, from which we can infer that iNOS may play a crucial role in vasospasm. An antioxidant-responsive element and a nuclear factor-κB response element have been found on the promoter region of the iNOS gene and these two elements are responsive to oxidative states. Previous reports have indicated that superoxide may play a vital role in the delayed onset of vasospasm in SAH. From the present study, we know that E2 treatment significantly diminishes SAH-induced iNOS mRNA expression. It is possible that the antioxidative property mediates the effect of E2 on the mRNA expression of iNOS via its antioxidative effect on these two elements. 

In the present study, the expression of iNOS mRNA and its protein were significantly elevated in the SAH group, which is in agreement with the results of previous studies. This finding indicates that iNOS was induced in the smooth muscle cells of cerebral blood vessels, endothelial cells, adventitial cells, and glial networks after SAH. Inhibition of iNOS has been shown to decrease cerebral vasospasm after SAH in rats, from which we can infer that iNOS may play a crucial role in vasospasm. An antioxidant-responsive element and a nuclear factor-κB response element have been found on the promoter region of the iNOS gene and these two elements are responsive to oxidative states. Previous reports have indicated that superoxide may play a vital role in the delayed onset of vasospasm in SAH. From the present study, we know that E2 treatment significantly diminishes SAH-induced iNOS mRNA expression. It is possible that the antioxidative property mediates the effect of E2 on the mRNA expression of iNOS via its antioxidative effect on these two elements.

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Estrogen attenuation of SAH-induced vasospasm

ated with the occurrence of SAH. Previous reports have demonstrated that the use of oral contraceptive medicine, early menstruation, and the combined effect of several variables related to menstrual and reproductive history are significantly associated with an increased risk of SAH. Nevertheless, sexual differences in the outcome of SAH are controversial, and the influence of female sex hormones is unclear. Estrogens have been found to exert neuroprotective effects in models of ischemic stroke both in vitro and vivo. Simpson and coworkers demonstrated that hypertensive Caucasian men with a history of intravenous drug abuse have a high risk of unfavorable outcome following SAH. Kongable and colleagues found that although women presenting with SAH are older and harbor more aneurysms, the 3-month outcome for women and men who experience aneurysmal SAH is similar. On the other hand, Johnston and associates showed that age-adjusted mortality rates of SAH were 62% greater in women than in men. Levels of estrogen with bioavailability, not the total amount of estrogen level, may be related to an increased SAH risk. The arterial vasodilatory effect after estradiol treatment can be reduced by the addition of progesterin treatment. Further studies of endogenous female steroid medication and exogenous E2 replacement and their interaction with progesterone are needed to explain outcome following SAH.

Conclusions

Continuous treatment with physiological levels of E2 is effective in the prevention of vasospasm without reducing blood pressure in a two-hemorrhage rodent model of SAH. The beneficial effects of estrogen on delayed vasospasm may arise from preservation of eNOS expression and prevention of iNOS induction by SAH. The use of E2 may hold therapeutic promise in the treatment of cerebral vasospasm following aneurysmal SAH and merits future investigation.

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