Estradiol Regulates Angiopoietin-1 mRNA Expression Through Estrogen Receptor-α in a Rodent Experimental Stroke Model

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Background and Purpose — Female, compared with male, animals are protected from cerebral ischemic injury. Physiological concentrations of 17β-estradiol (E2) reduce damage in experimental stroke. E2 augments angiogenesis in reproductive organs and noncerebral vascular beds. We hypothesized that E2 protects brain in stroke through modulation of angiogenesis. We quantified molecular markers of angiogenesis and capillary density before and after unilateral middle cerebral artery occlusion (MCAO).

Methods — Female animals were ovariectomized, treated with 25 μg E2 or placebo implants, and subjected to 2-hour MCAO and 22 hours of reperfusion. Brain angiopoietin-1 (Ang-1), Ang-2, Tie-1, Tie-2, vascular endothelial growth factor (VEGF), VEGF R1, and VEGF R2 mRNA levels were determined by RNAse protection assays, and CD31-positive vessels were counted.

Results — E2, but not ischemia, upregulated cerebral Ang-1 mRNA by 49%. Capillary density was higher in the brains of E2-treated animals. In estrogen receptor-α knockout (ERKO) mice, E2-mediated induction of Ang-1 mRNA was absent relative to wild-type littermates.

Conclusions — These results suggest that E2 increases Ang-1 and enhances capillary density in brain under basal conditions, priming the MCA territory for survival after experimental focal ischemia. (Stroke. 2005;36:337-341.)

Key Words: angiogenesis ■ cerebrovascular accident ■ endothelial growth factors

Women are protected from stroke relative to men; this advantage is lost with menopause. In 2002, the combined estrogen–progestin arm of the Women’s Health Initiative was stopped because of lack of efficacy in cardiovascular disease prevention, including stroke. The estrogen-alone arm of the trial was discontinued because of increased stroke risk in hormone users. Therefore, data from animals and cultured cells are under intense scrutiny to elucidate biological pathways of the effects of 17β-estradiol (E2) in brain and cerebral vasculature. The cellular and molecular pathophysiology of cerebral ischemia is strongly influenced by gender and female sex steroids. Although the preventative role of E2 is unclear, it does reduce stroke sensitivity (ie, attenuates neuronal damage once ischemia occurs). The mechanism of protection is likely multifactorial. E2 signals through estrogen receptor-α (ERα) and ERβ to alter gene expression. Rodent brains express both receptors, but ERα functions in E2-mediated protection in stroke. A candidate mechanism of cerebral protection is the ability of E2 to modulate angiogenesis. E2-mediated angiogenesis occurs in normal tissues (eg, female reproductive tract, retina, and other nonreproductive tissues) as well as pathologic conditions (eg, experimental limb ischemia, tumors, and aging brain).

Enhancement of angiogenesis is an attractive property. Improvement of collateral blood flow through angiogenesis or other endothelial-based adaptive mechanisms may occur after transient ischemic attacks, before stroke, in patients with intracranial stenosis. Such patients may be protected from infarction after vessel closure, similar to phenomena in coronary vasculature. Angiogenesis also occurs after stroke and may improve outcome. Current imaging modalities are of insufficient resolution to visualize the smallest collateral vessels, and therefore, molecular markers of angiogenesis are sometimes used to provide spatial and temporal quantification of angiogenesis.
Several angiogenic factors are involved in the production and maintenance of vascular networks.31,32 The angiopoietin family includes angiopoietin-1 (Ang-1) and Ang-2.32 Ang-1 signals through the Tie-2 receptor, reducing vascular permeability and stabilizing blood vessels.32-34 Ang-2 competitively inhibits the Ang-1/Tie-2 interaction, leading to blood vessel disruption.35,36 Tie-1 modulates Tie-2 by direct intramembrane interactions.37 The vascular endothelial growth factor (VEGF) family includes angiopoietin-1 (Ang-1) and Ang-2.32 Ang-1 containing receptor [KDR] or VEGF R1 (flt-1) and VEGF R2 (kinase insert domain-containing receptor [KDR] or flk-1).39 The 2 families are linked functionally: the effect of VEGF is dependent on the Ang-1/Ang-2 ratio. When Ang-2 is abundant and the ratio is low, VEGF promotes robust remodeling of the vascular network.36

In this study, we investigated whether E2 modulates expression of angiogenesis markers and alters brain capillary density in an established model of experimental stroke. Using reversible middle cerebral artery occlusion (MCAO),40 we evaluated the effect of E2 on angiopoietin and VEGF family gene expression and determined whether these transcriptional changes were mediated by ERα.

Materials and Methods

Middle Cerebral Artery Occlusion

All methods were described previously.41-44 Animal experiments were performed according to institutional guidelines. Female Wistar rats (weighing 200 to 250 g; Harlan Breeders) were ovariecotomized and subcutaneously implanted with placebo or E2 pellets (25 μg; 21-day release; Innovative Research of America). One week later, animals underwent sham surgery or 2-hour MCAO, followed by 22 hours of reperfusion. Animals were anesthetized with halothane (induction 2.5%; maintenance 1%); femoral arterial lines were placed for blood sampling and mean arterial pressure (MAP) monitoring; and the skull overlying the right MCA territory was thinned for laser Doppler flow (LDF) monitoring (Moor Instruments). A 4.0 mmol filament suture was introduced into the internal carotid artery and advanced until LDF dropped to <45% of baseline. MAP and rectal and temporalis temperatures were monitored and controlled, and arterial blood gases, blood glucose, and hemoglobin concentrations were monitored before occlusion and 1 hour into ischemia. After 2 hours of ischemia, the suture was removed and reperfusion documented by LDF. After 22 hours of reperfusion, the animal was euthanized, the brain removed, and blood obtained for E2 radioimmunoassay (Diagnostic Products).40 For mRNA quantification, brains were separated into hemispheres and frozen in 2-methylbutane (Sigma-Aldrich) on dry ice.

ERKO mice (weighing 22 to 26 g) were studied using a 2-hour MCAO protocol with a 6.0 suture.42 Seven days before ischemia, ERKO and age- and weight-matched wild-type (WT) mice were ovariecotomized and treated with subcutaneous E2 or vehicle (Silastic capsule; 0.062 ID, 0.125 OD), as described previously.42,43 Intrainschirnial neurologic deficit was scored as follows: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by the tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling.43.44 If no deficit was observed, the animal was removed from further study. After 22 hours of reperfusion, mice were processed as described above.

mRNA Quantification

RNA was extracted from brains using Trizol (Invitrogen) and chloroform. mRNA levels were determined by RNase protection assay (RPA; RPA III kit; Ambion) using previously characterized probes for Ang-1, Ang-2, Tie-1, and Tie-2.45 VEGF, VEGF R1, and VEGF R2 RNA probes were subcloned into the EcoRI/BamH1 site of pBluescript II SK (+) using the following primers: VEGF: sense 5′-AACAAAGCCGAAAAACAA-3′; antisense 5′-TCACGCGCTTGGCTTGTCACA-3′; VEGF R1: sense 5′-ACG GA-CAGTTAACAACAGGA-3′; antisense 5′-GACTCTTTC- AATAAACGCGT-3′; VEGF R2: sense 5′-CTCACACCAGT TTGCAGAA-3′; and antisense 5′-AGTACAATGCTAATCT-3′. Probes were labeled with 32P-UTP using T7 polymerase (Am- bion).45 After hybridization, samples were processed on polyacryl- amide gels, and mRNA species were quantified relative to actin using a phosphorimager (Molecular Dynamics).

Capillary Counts

Cerebral microvessels were counted in placebo- and E2-treated nonischemic and ischemic rats. Brains were removed, placed in embedding-medium-filled plastic molds, and frozen in 2-methylbutane on dry ice. In each animal, 8 5-μm-thick coronal sections were collected every 50 μm, ~1.7 mm to ~0.8 mm from bregma. The sampled area corresponded to plates 11 through 16 in Paxinos and Watson’s The Rat Brain in Stereotaxic Coordinates.46 Sections were processed with anti-CD31 antibody (Chemicon) at 1:100 dilution, and labeling was visualized with diaminobenzidine (DAB; Vector Laboratories). Three regions per hemisphere per slice, corresponding to basal ganglia, parasagittal cortex, and parietal cortex (Figure 1), were digitally photographed at ×200. DAB-positive structures were quantified using Metamorph Offline version 4.6r4 software (Universal Imaging). Capillary counts from all 8 slices were averaged to derive 1 capillary count number per region per animal. In nonischemic animals, left hemisphere capillary counts were quantified and compared between placebo- and E2-treated animals. In postischemic animals, ischemic/nonischemic hemisphere capillary count ratios were derived and compared between placebo and E2-treated animals.
**Results**

MAP, Pco₂, Po₂, and temperature were controlled; only animals with normal values were included in subsequent analyses. Ischemic LDF was <45% of baseline in all rat protocols, confirming adequate occlusion. In mice, intraschomic neurologic deficit scores were similar in all groups (P=0.37; ANOVA): E2-treated ERKO mice 2.6±0.5; vehicle-treated ERKO mice 2.7±0.2; E2-treated WT mice 2.0±0; vehicle-treated WT mice 2.3±0.2. Plasma E2 levels were 4±1 pg/mL in placebo-treated and 19±1 pg/mL in E2-treated rats (P<0.001), consistent with metestrus/diestrus values. In WT mice, plasma E2 was 18±3 pg/mL and 263±79 pg/mL in vehicle- and E2-treated animals, respectively (P=0.021). In ERKO mice, plasma E2 was 14±1 pg/mL and 344±239 pg/mL in vehicle- and E2-treated animals, respectively (P=0.295).

**E2 Selectively Increases Gene Expression in the Angiopoietin Family Through ERα**

Ang-1 mRNA was induced by E2 but not by ischemia (Figure 2). In E2-treated rats, Ang-1 mRNA levels were decreased in ischemic hemispheres. Ang-2 mRNA was induced by ischemia but not by E2 (Figure 3). Other angiopoietin and VEGF family members were not affected by E2 or ischemia (Table 1). To determine whether Ang-1 induction was mediated by ERα, we evaluated Ang-1 expression in ERKO mice. E2 increased Ang-1 expression in WT ovariectomized mice. However, E2-mediated Ang-1 expression was not observed in ERKO females (Figure 4).

**Brain Capillary Density Is Higher in Rats Treated With E2**

The brain regions selected for study (basal ganglia and parasagittal and parietal cortex) are directly involved in, or are in proximity to, regions injured in MCAO (Figure 1). In nonischemic rats, E2 treatment resulted in increased capillary density in the basal ganglia and parietal cortex but not in the parasagittal cortex (Table 2). Ischemic hemisphere/nonischemic hemisphere capillary density ratio in postschismic rats was 1 in placebo-treated and E2-treated animals 22 hours after stroke (data not shown).

**Discussion**

This study demonstrates 3 important findings. First, therapy with E2 selectively increases Ang-1 mRNA in ovariectomized female rat brain. Second, induction of Ang-1 is mediated by ERα. Third, capillary density is higher in brains of nonischemic animals treated with E2 compared with placebo. Ang-1 stabilizes microvascular networks and reduces vascular permeability. We speculate that E2-mediated augmentation of Ang-1 expression and enhanced microvas-
Ang-1.34 capillary density in young adult rodent brain before ischemia
E2 are ER
dent,51 and it is plausible that the metestrus plasma E2 levels
VEGF response to E2. VEGF induction is E2 dose depen-
sensitive to E2 and ischemia.8,9,39,51,52 We did not detect a
vascular structure and function before experimental stroke may
prime the brain for survival after stroke.
E2 modulates angiogenesis (ie, alters microvascular
network anatomy, endothelial cell function, and expression of
angiogenesis markers) in uterine and nonreproductive tis-
ues.8 E2 enhances endothelial cell proliferation, migration,
and survival in vitro.48,49 Some of the angiogenic effects of
E2 are ERα dependent.50 In the brain, E2 enhances cerebral
capillary density in senescent animals.14 Our finding that E2
increases brain Ang-1 mRNA through ERα and enhances
capillary density in young adult rodent brain before ischemia
compliments this large body of evidence. The higher capillary
density in E2-treated animals may result from new capillary
generation or stabilization of existing capillaries through
Ang-1.34
Numerous reports indicate that VEGF transcription is
sensitive to E2 and ischemia.8,9,39,51,52 We did not detect a
VEGF response to E2. VEGF induction is E2 dose depend-
ent,51 and it is plausible that the metestrus plasma E2 levels
in the rats were not sufficiently high to affect VEGF expres-
sion. We also did not observe a VEGF response to ischemia.
We may have missed the peak of induction because VEGF
increases at 3 hours after cerebral ischemia.27,51 Timing may
also explain our not detecting ischemic induction of Tie-1 and
Tie-2.29 An additional explanation is the low sensitivity of our
approach for detecting molecular changes in small brain
regions (eg, the ischemic penumbra). By harvesting mRNA
from the entire hemisphere, we dilute the mRNA, and only
the most robust changes, such as the ischemic induction of
Ang-2, are detectable.29,54
E2-mediated angiogenesis and protection in stroke are
absent in ERKO mice.7,55 Our finding that E2-mediated
induction of Ang-1 is attenuated in ERKO mice suggests that
this angiogenic factor is important for the ability of the
steroid to promote or maintain vascularization and may be an
important link between E2, angiogenesis or vascular stability,
and protection from stroke. Zhang et al introduced Ang-1
acutely into male mice and observed attenuated cerebral
edema because of a reduction in vascular permeability and
decreased stroke size.53 We hypothesize that chronic E2
treatment before stroke stabilizes or augments the cerebral
vasculature via elevation of Ang-1 and enhancement of tissue
resistance to ischemic damage through augmentation of
perfusion within the ischemic territory during experimental
stroke.40
Based on the results of the current experiments, Ang-1
emerges as a candidate molecule for E2-mediated neuropro-
tection in experimental transient focal cerebral ischemia. This
hypothesis must be tested directly because the results may be
applicable to the clinical arena, specifically to patients with
symptomatic intracranial vascular stenosis not amenable to
standard treatments.

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