Estrogen suppresses IL-1β-mediated induction of COX-2 pathway in rat cerebral blood vessels

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Ospina, Jose A., Holly N. Brevig, Diana N. Krause, and Sue P. Duckles. Estrogen suppresses IL-1β-mediated induction of COX-2 pathway in rat cerebral blood vessels. Am J Physiol Heart Circ Physiol 286: H2010–H2019, 2004.—Interleukin (IL)-1β is a potent inducer of inflammatory prostaglandins, which are important mediators of vascular response to cerebral injury, whereas estrogen reduces brain injury in models of ischemic stroke. Thus we examined the effects of in vivo IL-1β exposure on cerebrovascular cyclooxygenase (COX)-2 expression and function in an animal model of chronic estrogen replacement. Estrogen-treated and nontreated ovariectomized female rats received IL-1β injections (10 μg/kg ip), and then cerebral vessels were isolated for biochemical and contractile measurements. In estrogen-deficient rats, IL-1β induced cerebrovascular COX-2 protein expression; a peak response occurred 3 h after injection. COX-2 was localized to arterial endothelium using confocal microscopy. IL-1β increased PGE2 but not PGI2 production and decreased vascular tone as measured in isolated cerebral arteries; the latter effect was partially reversed by treatment with the selective COX-2 inhibitor NS-398 (10 μm). In contrast, in animals treated with estrogen, IL-1β had no significant effect on COX-2 protein levels, PGE2 production, or vascular tone. Combined treatment with 17β-estradiol and medroxyprogesterone acetate also prevented increases in PGE2 production after IL-1β treatment, but treatment with 17α-estradiol had no effect. IL-1β induction of COX-2 protein was prevented by treatment with the nuclear factor-κB inhibitor caffic acid phenethyl ester (20 mg/kg ip), and estrogen treatment reduced cerebrovascular nuclear factor-κB activity. Estrogen thus has potent anti-inflammatory effects with respect to cerebral vascular responses to IL-1β. These effects may have important implications for the incidence and severity of cerebrovascular disease.

17β-estradiol; cyclooxygenase-2; prostaglandin E2; nuclear factor-κB; interleukin-1β

ESTROGEN ATTENUATES ischemia-reperfusion-related cerebral injury in experimental animal models of stroke (23, 53). Emerging evidence indicates that the neuroprotective mechanisms afforded by estrogen are multifaceted, complex, and dependent on cell type. Although most efforts have focused on the effects of estrogen on neurons and glia, the cerebral vasculature is an important target given that it is the initial site of cellular inflammation in the brain after focal ischemia (9), and its function is regulated by estrogen during nonpathological conditions (18, 19, 37, 38).

Cerebrovascular inflammation is an early event in the pathogenesis of stroke and is believed to contribute to secondary brain injury by promoting leukocyte infiltration into the brain (16), blood-brain-barrier injury (10), hyperemia (23), vaso-
genic edema, and increased intracranial pressure (12). Central to the initiation of inflammation is the synthesis of cytokines that signal the induction of proinflammatory mediators primarily through activation of nuclear factor-κB (NF-κB), a family of transcription factors that are ubiquitously involved in the coordinated expression of inflammation-related genes (28). In the central nervous system, interleukin (IL)-1β appears to be the predominant cytokine (12) and is released from multiple cell types, including astrocytes, neurons, microglia, and endothelial cells (39). It has been suggested (40) that IL-1β is an important contributor to the pathogenesis of stroke, because inhibition of IL-1β has been shown to reduce neurodegeneration after ischemic injury. However, a thorough understanding of the influence of IL-1β on cerebral circulation is lacking.

Cyclooxygenase-2 (COX-2) is an important mediator of vascular response to injury, and this enzyme is induced in response to cytokines, lipoplysaccharide (LPS), and ischemia-reperfusion (7). Once induced, COX-2 catalyzes the conversion of arachidonic acid to inflammatory prostaglandins, predominantly PGE2 and PGI2 (11). Vascular-derived prostanooids may be important mediators of cerebral vascular inflammation in that cyclooxygenase products promote cell adhesion molecule expression (46), postischemic hyperemia (31), blood-brain-barrier leakiness (51), and cytotoxicity (43). Conversely, estrogen can suppress leukocyte accumulation (41) and attenuate hyperemia (23) in the brain after an inflammatory insult. It was recently shown (17) that estrogen can suppress NF-κB-dependent inflammation in cultured cerebral endothelial cells; however, a similar effect of estrogen in vivo has yet to be demonstrated. Taken together, these observations raise the possibility that estrogen suppresses cerebrovascular inflammation by regulating proinflammatory prostaglandin synthesis and function. Consequently, estrogen may confer benefit against ischemia-reperfusion brain injury by minimizing functional dysregulation of the cerebral circulation.

Therefore, in this study we examined the effects of in vivo IL-1β administration on COX-2 pathway expression, localization, and function in cerebral blood vessels of estrogen-deficient and -treated female rats. The latter group, ovariectomized females chronically treated with 17β-estradiol, also was compared with animals treated with either the inactive enantiomer 17α-estradiol or the clinically used combination of 17β-estradiol and medroxyprogesterone acetate (MPA). In addition, we explored the role of NF-κB in mediating induction of cerebrovascular COX-2 by IL-1β and whether its activation is influenced by in vivo 17β-estradiol treatment. To our knowledge, this is the first study to provide evidence that 1) estrogen...
regulates cytokine-mediated induction of cerebrovascular inflammatory prostaglandin pathways in vivo, 2) NF-κB participates in the induction of cerebrovascular COX-2 after peripheral IL-1β administration, and 3) chronic 17β-estradiol treatment regulates vascular NF-κB activation. These results suggest that in vivo estrogen treatment, by targeting NF-κB activation, suppresses the inflammatory drive created by COX-2-derived products after exposure to inflammatory stimuli.

**MATERIALS AND METHODS**

*In vivo hormone treatment.* All protocols involving the use of animals were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine. Four groups of female rats were used in this study: ovariectomized (OVX), OVX treated with 17β-estradiol (OVX+17β-E), OVX treated with 17α-estradiol (OVX+17α-E), and OVX+β-E treated with MPA (OVX+β-E+MPA). Three-month-old female Fischer 344 rats (Charles River-SASCO Laboratories; Wilmington, MA) were ovariectomized while under anesthesia (46 mg/kg ip ketamine and 4.6 mg/kg ip xylazine). At the time of ovariectomy, some rats were subcutaneously implanted with Silastic capsules (inner diameter, 1.57; outer diameter, 3.18 mm) that contained either 17β-estradiol (length, 5 mm), 17α-estradiol (5 mm), or 17β-estradiol (5 mm) and MPA (3 mm). Animals were allowed to recover from surgery and then were returned to a vivarium where they were housed in individual cages with free access to food and water in a temperature-controlled (22°C) room on a 12:12-h light-dark cycle.

Approximately 1 mo after surgery and hormone capsule implantation, animals were injected with IL-1β (10 μg/kg ip) or an equivalent volume of vehicle (0.9% saline ip). In some cases, OVX animals also received injections of 20 mg/kg ip caffeic acid phenethyl ester (CAPE; BIOMOL; Plymouth Meeting, PA) or vehicle [polyethylene glycol (PEG)-400] at 24, 12, and 1 h before IL-1β or saline treatment. At various time points (20, 60, 180, or 360 min) after IL-1β or saline injection, animals were anesthetized by CO2, and blood samples were obtained by cardiac puncture for measurement of serum 17β-estradiol by radioimmunoassay (Diagnostic Products; Los Angeles, CA). Alternatively, serum IL-1β levels were determined by ELISA (Assay Designs; Ann Arbor, MI). Although MPA levels were not quantitated, our lab has previously demonstrated that implantation of 3-mm-long MPA capsules in female rats results in MPA serum levels that are significantly different from OVX rats treated with 17β-estradiol (OVX+17β-E).

Provided in Table 1 are the statistical differences measured in vivo in the concentrations of 17β-estradiol, body weight, and uterine weight in female rats treated with estrogen. Our lab has previously demonstrated that implantation of 3-mm-long 17β-estradiol (length, 5 mm) and MPA (3 mm) capsules in female rats results in MPA serum levels that are significantly different from OVX rats treated with 17β-estradiol (OVX+17β-E).

**Table 1. Effects of treatment on 17β-estradiol serum concentration, body weight, and uterine weight**

<table>
<thead>
<tr>
<th>Rat Group</th>
<th>n</th>
<th>17β-Estradiol, pg/ml</th>
<th>Body Wt, g</th>
<th>Uterine Wt, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX</td>
<td>27–54</td>
<td>6.2 ± 1</td>
<td>200 ± 2</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>OVX+17β-E</td>
<td>29–43</td>
<td>56 ± 2*</td>
<td>176 ± 1*</td>
<td>80 ± 2</td>
</tr>
<tr>
<td>OVX+17α-E</td>
<td>7 or 8</td>
<td>9.2 ± 1*</td>
<td>190 ± 4 حت</td>
<td>28 ± 2 حت</td>
</tr>
<tr>
<td>OVX+β-E+MPA</td>
<td>8</td>
<td>45 ± 8*</td>
<td>179 ± 2*</td>
<td>69 ± 2*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. Statistical differences were determined by one-way ANOVA and subsequent Newman-Keuls post hoc tests. αE, 17α-estradiol; MPA, medroxyprogesterone acetate. *Significantly different from ovariectomized (OVX) rats; †P < 0.05, significantly different from OVX rats treated with 17β-estradiol (OVX+17β-E).
Prostanoid measurement. Prostanoid release from cerebral vessels of OVX, OVX+βE, OVX+αE, and OVX+βE+MPA animals was measured after a 3-h in vivo treatment with IL-1β or saline. Freshly isolated brains were gently homogenized and washed in ice-cold 0.01 mol/l PBS (see Western blot analysis). Brain homogenates were passed over a 150-μm nylon mesh to separate the blood vessels (retained) from the rest of the brain tissue (washed through). After extensive washing, the vessels were collected and placed into HEPES-buffered saline (HBS, pH 7.4) that contained (in mmol/l) 122 NaCl, 1.6 CaCl₂, 25.6 NaHCO₃, 5.2 KCl, 1.2 KH₂PO₄, and 11.5 dextrose (all from Fisher Scientific; Pittsburgh, PA) and equilibrated in a tissue-culture incubator (at 37°C in 95% O₂-5% CO₂) for 1 h. After the equilibration period, tissues were placed into fresh HBS at 37°C (in 95% O₂-5% CO₂) and incubated for 30 min. Separate aliquots of cerebral vessels from each brain were incubated in the presence and absence of 10 μmol/l NS-398 (Cayman Chemical) to assess the specific contribution of COX-2 to prostanoid production. After incubation, the medium was collected and immediately stored frozen at −20°C until prostanoid measurement. Tissues were lysed and protein contents were determined via BCA assay. Enzyme immunoassay was used to measure PGE₂ directly (Assay Designs) or to measure PGF₁α as its stable metabolite 6-keto-PGF₁α (Amersham) according to the protocols provided by the manufacturers. PGE₂ and 6-keto-PGF₁α values were normalized to tissue protein concentrations. COX-2-derived PGE₂ production quantities were determined as the difference in PGE₂ levels obtained from tissues incubated in the absence and presence of 10 μmol/l NS-398.

Contractile studies of pressurized arteries. Vascular tone was measured in cerebral arteries from OVX and OVX+βE rats killed 3 h after injection of IL-1β or saline. Freshly isolated brains were immediately placed in ice-cold physiological saline solution (PSS) that contained (in mmol/l) 122 NaCl, 1.6 CaCl₂, 25.6 NaHCO₃, 5.2 KCl, 1.2 KH₂PO₄, 0.03 EDTA, and 11.5 dextrose and were bubbled with 95% O₂-5% CO₂. Using a dissecting microscope, we isolated a 2- to 3-mm segment of a small-caliber branch of the middle cerebral artery from the area overlying the parietal cortex. Arteries with a passive intraluminal diameter of ~130–140 μm at a pressure of 80 mmHg were chosen for this study. Once isolated, vessels were cleaned of any adhering tissue and placed into an arteriograph that contained (in mmol/l) 122 NaCl, 1.6 CaCl₂, 25.6 NaHCO₃, 5.2 KCl, 1.2 KH₂PO₄, 0.03 EDTA, and 11.5 dextrose and were bubbled with 95% O₂-5% CO₂. Using a dissecting microscope, we isolated a 2- to 3-mm segment of a small-caliber branch of the middle cerebral artery from the area overlying the parietal cortex. Arteries with a passive intraluminal diameter of ~130–140 μm at a pressure of 80 mmHg were chosen for this study. Once isolated, vessels were cleaned of any adhering tissue and placed into an arteriograph that contained PSS (Living Systems Instrumentation; Burlington, VT). One end of the artery was mounted and secured onto a glass microvessel, and pressure was set at 60 mmHg and allowed to equilibrate for 1 h while being superfused with PSS maintained at 37°C and pH 7.4 and bubbled with 95% O₂-5% CO₂. Vessels that did not develop spontaneous tone during the initial equilibration period or that had a passive diameter >150 μm at 80 mmHg were discarded. Intraluminal diameter and transmural pressure values were monitored by computer-based analysis using MacLab and Chart 3.6 software.

After equilibration, the autoregulatory response in cerebral arteries was assessed. Transmural pressure was decreased to 20 mmHg and diameter was allowed to stabilize over a 5- to 10-min period. Pressure was then gradually increased to 80 mmHg and the vessel was allowed to reach a stable diameter. Pressure was then returned to 60 mmHg and arteries were superfused with PSS that contained the selective COX-2 inhibitor NS-398 (10 μmol/l; Ref. 37) for 30 min. Pressure was again decreased to 20 mmHg and then gradually increased to 80 mmHg, and vessel diameters were recorded. At the conclusion of all experiments, vessels were superfused for 5–10 min with Ca²⁺-free PSS that contained 3 mmol/l EDTA, and the passive intraluminal diameters were measured at 20 and 80 mmHg. Vascular tone was defined as the difference between the passive (EDTA treated) and active (PSS or NS-398 treated) diameters. Autoregulatory response was determined as the difference in vascular tone between 80 and 20 mmHg.

NF-κB p65 measurement. NF-κB p65 DNA-binding activity was measured in nuclear extracts of cerebral vessels from OVX and OVX+βE rats killed 20 min after injection of IL-1β or saline. Nuclear and cytosolic extracts were prepared as described previously (20). Briefly, cerebral blood vessels were isolated, finely diced on a glass slide that was kept over dry ice, Dounce homogenized in ice-cold buffer A (that contained 10 mmol/l HEPES, 1.5 mmol/l MgCl₂, 10 mmol/l KCl, 1 mmol/l DTT, 1 mmol/l PMSF, and 0.1% Nonidet P-40, pH 7.9), and incubated on ice for 15 min. After homogenization, samples were centrifuged at 870 g for 10 min at 4°C. Pellets were resuspended in buffer A without Nonidet P-40 and recentrifuged at 870 g for 10 min at 4°C. Supernatants were discarded, and pellets were suspended in TransAm lysis buffer (Active Motif; Carlsbad, CA) and shaken for 30 min at 4°C. Suspensions were then microcentrifuged at 14,000 g for 10 min at 4°C. Supernatants that contained nuclear extracts were collected, and protein contents were determined via BCA assay.

NF-κB p65 DNA binding was measured in nuclear extracts with a TransAM NF-κB p65 immunoassay-based kit (Active Motif). Nuclear protein or HeLa whole cell extracts for positive control (2.5 μg/well) were incubated for 1 h in 96-well plates to which a double-stranded NF-κB consensus oligonucleotide sequence (5'-AGTGTAGGGGAC-TTTCCAGGC-3' and 3'-TCACACCCCTGAAGGGTGCC-5'; binding site underlined) had been conjugated (Active Motif). Activated NF-κB p65 was detected by 1-h incubation with an anti-p65 antibody that recognizes an epitope as accessible only when NF-κB is bound to DNA. This was followed by a 1-h incubation with a horse-radish peroxidase-conjugated secondary antibody and finally by exposure to a 3,3',5,5'-tetramethylbenzidine substrate solution. Reactions were measured by colorimetric methods, and data were expressed as the percent of the positive control signal.

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed with GraphPad Prism 2.0 software. Differences were assessed by one-way ANOVA or ANOVA with repeated measures where appropriate. Pairwise comparisons were made using Newman-Keuls post hoc analysis. Alternatively, Student’s t-test was used where appropriate. For all comparisons, statistical significance was set at P ≤ 0.05.

RESULTS

In vivo model of hormone treatment. We used an in vivo rat model to explore the effects of chronic estrogen deficiency (OVX) or chronic treatment with 17β-estradiol (OVX+βE), 17α-estradiol (OVX+αE), or 17β-estradiol plus MPA (OVX+βE+MPA) on the induction of cerebrovascular COX-2 pathways after peripheral IL-1β treatment. To verify our model, serum 17β-estradiol levels, whole body weights, and dry uterine weights were measured 1 mo after surgical treatment (Table 1). Serum from OVX+βE or OVX+βE+MPA rats displayed levels of 17β-estradiol that approximated those found in normally cycling rats (29); these levels were significantly greater than in OVX animals. Animals treated with 17α-estradiol had serum 17β-estradiol levels similar to those detected in OVX rats. In addition, OVX+βE and OVX+βE+MPA rats had significantly lower whole body weights and greater uterine weights compared with OVX rats, although MPA attenuated the stimulatory effect of estrogen on uterine growth. Treatment with 17α-estradiol resulted in a small decrease in body weight and a slight increase in uterine weight; both measures were significantly different than those found in 17β-estradiol-treated rats. These results suggest that implanted pellets sustained the physiological levels of 17β-estradiol.
estriol in the serum throughout the treatment period. Furthermore, circulating 17β-estriol was biologically active, and its trophic effects on the uterus were partially antagonized by MPA.

**IL-1β induction of cerebrovascular COX-2.** We first determined the effects of circulating IL-1β on the cerebrovascular COX-2 pathway in OVX+βE and OVX rodents. We initially examined the time course of COX-2 protein expression in cerebral vessels for the 6-h period after peripheral administration of IL-1β. Western blots for cerebral vessels from OVX and OVX+βE animals treated with IL-1β were probed with anti-COX-2 polyclonal antibodies, and bands migrating at 72 kDa were detected (Fig. 1). These bands corresponded to the expected molecular weight of COX-2 and comigrated with the positive control used. COX-2 protein was detectable 1 h after IL-1β treatment; however, at this time point, the levels of COX-2 were not different between OVX and OVX+βE animals. At 3 and 6 h after IL-1β exposure, the levels of COX-2 protein were significantly increased in vessels from OVX animals (Fig. 1). In contrast, in OVX+βE animals, levels of cerebrovascular COX-2 remained unchanged at 3 and 6 h after cytokine injection (Fig. 1). COX-2 levels were thus significantly different between OVX and OVX+βE rats at both 3 and 6 h after IL-1β treatment.

**COX-2 localization in cerebral arteries after IL-1β treatment.** Laser scanning confocal microscopy was used to determine the cellular localization of COX-2 in cerebral arteries isolated from OVX rats 3 h after IL-1β injection. Using the orientation of DAPI-stained nuclei as a guide (2), we identified the smooth muscle cell layer by nuclei oriented perpendicular to the direction of blood flow (Fig. 2A) and the endothelial cell layer by nuclei oriented in the direction of blood flow (Fig. 2B). Once the vascular cell layer was identified, immunofluorescent staining for COX-2 (green) and the endothelial marker eNOS (red) was examined in the same focal plane. The eNOS was visualized in the endothelium (Fig. 2D) but not the smooth muscle cells (Fig. 2E). Merging all three images at the endothelial level (Fig. 2H) illustrates a partial colocalization of eNOS and COX-2 adjacent to the nuclei. In contrast, merging of images taken at the level of cerebral artery smooth muscle (Fig. 2E) demonstrates no localization of eNOS or COX-2. We previously found no detectable fluorescence when the secondary antibodies were used alone (C. Stirene, S. P. Duckles, and D. N. Krause, unpublished observation).

**Estrogen regulation of IL-1β induction of COX-2.** We next compared the effects of vehicle (0.9% saline) and IL-1β on cerebrovascular COX-2 induction 3 h after injection, as at this time the effects of IL-1β were most salient. As shown in Fig. 3, COX-2 band densities were expressed at very low yet similar levels after treatment of OVX or OVX+βE animals with saline. Administration of IL-1β, however, significantly increased COX-2 levels in OVX rats. In contrast, there was no cytokine effect in OVX+βE animals. As expected, α-actin levels were similar between all treatment groups.

**Effects of estrogen on serum IL-1β.** Estrogen does not appear to influence the in vivo degradation of IL-1β in that there were no significant differences in serum IL-1β concentrations between OVX (3.8 ± 0.9 ng/ml; n = 3) and OVX+βE (2.9 ± 1.9 ng/ml; n = 4) animals 20 min after IL-1β injection.

**Effects of estrogen, MPA, and IL-1β on cerebrovascular PGE2 production.** We next assessed whether treatment of rats with IL-1β stimulated the elaboration of inflammatory prostaglandins, primarily PGE2, from cerebral vessels incubated in vitro, and if so, whether prostanoid levels were influenced by prior chronic in vivo treatment with 17β-estradiol, 17α-estradiol, or 17β-estradiol and MPA. Consistent with our previous study, there were no significant differences in PGE2 levels among the saline-treated groups (38). Figure 4 illustrates that prior in vivo IL-1β treatment significantly increased in vitro cerebrovascular PGE2 release from vessels of OVX animals compared with vessels from saline-treated controls. Chronic in vivo 17β-estradiol treatment, however, prevented the inductive effects of IL-1β on cerebrovascular COX-2-derived PGE2, and this preventive effect of 17β-estradiol was preserved in vessels from animals simultaneously treated with MPA. On the other hand, when ovariectomized rats were chronically treated with 17α-estradiol (which is the inactive enantiomer of 17β-estradiol), IL-1β significantly stimulated cerebrovascular COX-2-derived PGE2 production to levels comparable to those seen in vessels from OVX animals. Consequently, in vitro cerebrovascular PGE2 production 3 h after in vivo IL-1β exposure was significantly greater in vessels from OVX and OVX+βE but
However, did not reach statistical significance of OVX animals did not increase cerebrovascular pro-
not OVX+βE+MPA animals compared with OVX+βE rats (Fig. 4).

Because COX-2 is believed to be a major source of prosta-
cyclin in vascular cells, we investigated the possibility that
IL-1β upregulates prostacyclin release. However, IL-1β treat-
ment of OVX animals did not increase cerebrovascular pros-
tacyclin production. Rather, there was a trend toward decreased
 release of PGI2 in vessels from IL-1β-treated animals (518 ±
120 pg/mg protein; n = 3) compared with saline-treated
controls (910 ± 127 pg/mg protein; n = 3). These differences,
however, did not reach statistical significance.

Effects of estrogen and IL-1β on tone of cerebral arteries.
Next we determined the effects of peripheral IL-1β or saline
administration to OVX and OVX+βE animals on tone and
autoregulation of pressurized small-caliber cerebral arteries
isolated from these animals. As shown in Fig. 5A, 3 h after
IL-1β administration to OVX rats, vascular tone in isolated
cerebral arteries pressurized to 80 mmHg was significantly
reduced compared with arteries isolated from saline-treated
OVX rats. In contrast, in arteries from OVX+βE rats, there
were no differences between saline or IL-1β treatment with
respect to cerebrovascular tone at 80 mmHg. However, cere-
bral vascular tone in both OVX+βE groups was significantly
greater than the OVX IL-1β-treated group. Furthermore, in

support of our previous work (37), arteries from OVX+βE rats
(saline treated) displayed significantly less tone than those
from OVX saline-treated animals. At 80 mmHg, passive cere-
bral artery diameter measurements were nearly identical be-
tween saline- and IL-1β-treated OVX and OVX+βE animals
(Fig. 5B).

We also examined the changes in tone across a physiologi-
ical range of intraluminal pressures. Figure 5C illustrates that
increasing pressure from 20 to 80 mmHg resulted in increased
tone that was not significantly different in magnitude between
arteries from saline-treated OVX and OVX+βE rats. In addition,
there were no significant effects of IL-1β treatment on the
autoregulatory response of cerebral arteries from either OVX
or OVX+βE animals (Fig. 5C).

Role of COX-2 in IL-1β effects on cerebrovascular tone. The
contributions of COX-2 activity to IL-1β-mediated changes in
vascular tone were evaluated in pressurized cerebral arteries
from OVX and OVX+βE animals. As shown in Fig. 6A, in
cerebral arteries from OVX animals treated with IL-1β, selec-
tive pharmacological inhibition of COX-2 with NS-398 signif-
ically increased tone and thus partially reversed the decrease
in tone consequent to IL-1β treatment. Surprisingly, NS-398
caused a slight yet significant decrease in tone in cerebral
arteries from OVX animals treated with saline (Fig. 6A). In

Fig. 2. Laser scanning confocal microscopic localization of COX-2 in a
cerebral artery from an OVX rat 3 h after treatment with IL-1β. A, C, and E:
a single focal plane through the smooth muscle cell layer was imaged for either
nuclei (blue), endothelial nitric oxide synthase (eNOS, red), or COX-2 (green),
respectively. G: merged image of A, C, and E demonstrates no eNOS or
COX-2 staining at the level of arterial smooth muscle. B, D, and F: a different
focal plane through the endothelial cell layer of the same artery illustrates
nuclear, eNOS, and COX-2 staining, respectively. H: merged image of B, D,
and F shows colocalization of eNOS and COX-2 in the endothelium of
cerebral arteries.

Fig. 3. Effects of 17β-estradiol treatment on cerebrovascular COX-2 protein
levels 3 h after IL-1β or saline control injection. A: a representative Western
blot illustrates differences in COX-2 immunoreactivity in cerebral vessel
lysates from OVX and OVX+βE animals 3 h after IL-1β or saline adminis-
tration. Bands migrating at 72 and 42 kDa were detected using antibodies
directed against COX-2 and α-actin, respectively. B: COX-2 densitometric
data are illustrated as fold changes vs. OVX saline values. Values are means ±
SE; n = 3 rats; *P ≤ 0.05, significantly different.
Cerebral vessels released PGE$_2$ into the incubation medium, which was collected after 30 min. PGE$_2$ was measured by enzyme immunoassay and normalized to the amount of protein in tissue samples. COX-2-derived PGE$_2$ production was determined as the difference in PGE$_2$ levels obtained from tissue incubated in the absence and presence of 10 μmol/l NS-398. Values are means ± SE; n = 4–6 rats; *P ≤ 0.05, significantly different from saline in same animal group; #P ≤ 0.05, significantly different from OVX + βE IL-1β.

contrast, cerebral arteries from OVX + βE animals that received saline or IL-1β were unaffected by NS-398 (Fig. 6B).

Role of NF-κB in IL-1β induction of cerebrovascular COX-2: regulation by estrogen. We next explored whether NF-κB signaling was involved in induction of cerebrovascular inflammatory prostanooid pathways after peripheral IL-1β administration. Figure 7 depicts the effects of in vivo pretreatment of OVX rats with the selective NF-κB inhibitor CAPE (26, 33) before IL-1β injection. OVX animals treated with PEG-400 (the vehicle for CAPE) during the 24-h period preceding IL-1β administration expressed COX-2 in the cerebral vasculature at levels similar to those reported above (Fig. 7). CAPE (20 mg/kg) treatment for 24 h, however, abrogated the inductive effect of IL-1β on COX-2 and resulted in significantly lower levels of COX-2 protein expression.

We therefore hypothesized that estrogen regulates cerebrovascular COX-2 induction by suppressing NF-κB pathway activity. To test this hypothesis, NF-κB p65 DNA binding activity was measured in nuclear extracts of cerebral vessels isolated from OVX and OVX + βE animals treated for 20 min with IL-1β or saline. As shown in Fig. 8, levels of NF-κB p65 DNA binding were not different in cerebral vessels from OVX and OVX + βE animals after saline treatment. Nuclear NF-κB levels were also similar between saline-treated (OVX, 24 ± 6 vs. OVX + βE, 16 ± 6%) and uninjected (OVX, 15 ± 4 vs. OVX + βE, 12 ± 4%) animals. Interestingly, IL-1β injection significantly increased NF-κB p65 DNA-binding activity in cerebrovascular tissue from both OVX and OVX + βE animals compared with saline controls (Fig. 8). However, NF-κB levels were significantly lower in cerebrovascular nuclear extracts from OVX + βE vs. OVX animals treated with IL-1β (Fig. 8).

Fig. 4. Effects of 17β-estradiol (OVX+βE), 17α-estradiol (OVX+αE), or 17β-estradiol and medroxyprogesterone acetate (OVX+βE+MPA) treatment on cerebrovascular production of PGE$_2$ after 3 h in vivo treatment with IL-1β. Cerebral vessels released PGE$_2$ into the incubation medium, which was collected after 30 min. PGE$_2$ was measured by enzyme immunoassay and normalized to the amount of protein in tissue samples. COX-2-derived PGE$_2$ production was determined as the difference in PGE$_2$ levels obtained from tissue incubated in the absence and presence of 10 μmol/l NS-398. Values are means ± SE; n = 4–7 rats; *P ≤ 0.05, significantly different from OVX + βE IL-1β.

Fig. 5. Effects of chronic estrogen treatment on vascular tone, passive diameter, and autoregulatory response of small-caliber cerebral arteries from animals treated with IL-1β or saline. A: vascular tone at 80 mmHg intraluminal pressure in cerebral arteries isolated from OVX and OVX + βE rats 3 h after peripheral injection of IL-1β or saline. Tone was calculated as the difference between passive (determined in 3 mmol/l EDTA-Ca$^{2+}$-free physiological saline solution (PSS)) and active (determined in PSS) diameters. B: passive intraluminal diameters determined in 3 mmol/l EDTA-Ca$^{2+}$-free PSS. C: autoregulatory response was defined as the change in vascular tone between the application of 20 and 80 mmHg transmural pressure. In each case, vascular tone was calculated as the difference between passive (determined in 3 mmol/l EDTA-Ca$^{2+}$-free PSS) and active (determined in PSS) diameters. Values are means ± SE; n = 4–7 rats; *P ≤ 0.05, significantly different from OVX saline treatment; #P ≤ 0.05, significantly different from OVX IL-1β treatment.
DISCUSSION

Inflammatory prostaglandins are important mediators of the vascular response to cerebral injury and are believed to contribute to the pathogenesis of ischemic stroke. In this study, we demonstrate that in vivo cytokine induction of the inflammatory COX-2 pathway in cerebral vessels is dramatically suppressed by estrogen treatment. In estrogen-deficient female rats, the endothelium of cerebral arteries responded to circulating IL-1β with expression of COX-2 protein that correlated with increased PGE2 production and decreased vascular tone. This IL-1β-mediated induction of COX-2 was dependent on NF-κB activation. In contrast, after chronic estrogen replacement, cerebrovascular COX-2 expression, PGE2 production, and cerebral vascular tone were all unaffected by IL-1β administration. Chronically circulating estrogen thus has potent anti-inflammatory effects with respect to the cerebral vascular response to IL-1β administration. Estrogen modulation of cerebrovascular inflammatory prostanoid pathways may contribute to the ability of female gonadal steroids to reduce brain damage after ischemia (24, 53).

IL-1β is one of the primary cytokines produced after ischemic brain insult (9). In our study, the cytokine was injected peripherally because this has been shown to induce COX-2 protein expression in cerebrovascular tissue (4). IL-1β caused an induction of COX-2 expression in cerebral vessels of the estrogen-deficient rat; a peak response occurred 3 h after treatment but persisted through 6 h. In arteries, COX-2 was localized in the endothelial cell layer. Using cultured cells, others have shown a similar time course of COX-2 expression after exposure to IL-1β (35, 51) as well as other cytokines (27).

The present study focused on the effects of estrogen on the inflammatory response to IL-1β; therefore, we compared estrogen-deficient with chronically estrogen-treated females. Additional studies are necessary to explore the effects of gender. However, preliminary studies suggest a similar correlation with estrogen levels in male and female rats as well as throughout the course of the estrus cycle (L. Sunday, S. P. Duckles, and D. N. Krause, unpublished observations). We demonstrated that induction of COX-2 by IL-1β was suppressed by prior treatment with estrogen. It is possible that PGE synthase is also induced by IL-1β; however, additional
estrogen reduced IL-1β-pial artery autoregulatory response. These findings treated animals. Neither estrogen nor IL-1β/H9252/H11001/H9252/OVX.

Our findings suggest that the induction of additional vasodilators unrelated to COX-2. The most likely explanation is that IL-1β also induces inductive nitric oxide synthase expression, which would produce the potent vasodilator nitric oxide (9, 36, 40). In fact, preliminary studies from our laboratory suggest that this is the case (L. Sunday, S. P. Duckles, and D. N. Krause, unpublished observations).

An unanticipated effect was the slight decrease in tone observed when arteries from saline-treated OVX rats were exposed to NS-398. We found that COX-2 is present, albeit at low levels, in cerebral vessels of saline-treated OVX rats; this would result in the synthesis of prostaglandin endoperoxide (PGH2) from arachidonic acid. Although PGH2 is typically converted to any one of the prostanooids by prostaglandin-specific synthases, our work (37) as well as that of others (8) indicates that in the absence of estrogen, the activity of an endogenous thromboxane-PGH2 receptor agonist speculated to be PGH2 is upregulated. Blockade of such a constrictor substance by NS-398 could account for the decrease of tone in arteries from saline-treated OVX rats.

The potential interplay of multiple cell and tissue types in the intact organism complicates the mechanistic interpretation of this in vivo study. Differences in metabolism of circulating IL-1β, however, do not account for the differential expression of COX-2 pathway activity because serum IL-1β levels were not affected by estrogen treatment. The expression of IL-1 receptors in cerebral vascular endothelium (15), the colocalization of COX-2 and IL-1 receptor mRNA in brain endothelial cells (4), and the rapid activation (20 min) of NF-κB demonstrated here all suggest that IL-1β is directly responsible for the induction of inflammatory prostaglandins in cerebral vessels. Therefore, signaling mechanisms downstream to the activation of IL-1 receptors may be possible targets for estrogen regulation.

Activation of transcription by NF-κB is critical in initiating the development of inflammatory processes. NF-κB is a transcriptional activator that is normally maintained inactive in the cytosol while complexed with the inhibitory protein IκBα (28). Inflammatory stimuli such as IL-1β cause degradation of IκBα and translocation of NF-κB dimers to the nucleus. There they bind κB consensus sequences and activate target gene transcription. In cultured cells, IL-1β has been shown to induce the transcription of COX-2 via NF-κB activation in some studies (5, 34) but not in others (50). Furthermore, the gene for COX-2 possesses two κB sites in its promoter (34). In our study, we provide evidence that peripheral IL-1β treatment induces cerebrovascular COX-2 protein expression via the activation of NF-κB.

In cell culture, estrogen inhibits the activation of NF-κB by preventing IκBα degradation (44). Alternatively, estrogen might act through a mechanism unrelated to degradation of IκBα (17), for example, by inhibiting NF-κB transport into the nucleus or reducing NF-κB binding activity. In our study, estrogen only partially suppressed NF-κB DNA-binding activity, whereas it completely inhibited IL-1β induction of COX-2 and PGE2 and thus raised the possibility that estrogen also.

studies are necessary to explore whether this enzyme is also suppressed by estrogen.

The ability of estrogen to suppress IL-1β induction of PGE2 production persisted when MPA treatment was combined with estrogen treatment for 1 mo. MPA is a common ingredient of combination estrogen-progestin hormone-replacement therapy, and some have speculated that addition of MPA may negate the beneficial cardiovascular actions of estrogen. Our finding that MPA does not block the suppressive actions of estrogen on PGE2 production is consistent with previous findings that MPA does not affect estrogen-induced increases in cerebrovascular eNOS (30) or suppression of ICAM-1 and E-selectin in cultured cells (32).

To better understand the physiological significance of inflammatory prostaglandin induction in the cerebral circulation, we measured contractile function of small-caliber branches of the middle cerebral artery. These were used because they are important regulators of cerebral vascular resistance and are situated downstream from the typical site of thromboembolic occlusion and thus contribute to postischemic hyperemia (51). Consistent with our previous studies (18, 37), we found that within the range of physiological pressures, saline-treated OVX rats displayed greater vascular tone than their estrogen-treated counterparts. Administration of IL-1β to OVX rats dramatically decreased isolated cerebral arterial tone, but there were no effects of IL-1β treatment on vascular tone in estrogen-treated animals. Neither estrogen nor IL-1β affected the pial artery autoregulatory response. These findings suggest that estrogen reduced IL-1β-mediated endothelial-dependent cerebral vasodilatation; this would perhaps be manifest as diminished hyperemia in the in vivo situation (23). Based on the results of others (3, 5, 31, 36), we suspected that the IL-1β-mediated decrease in cerebrovascular tone was perhaps governed by COX-2-derived PGE2. The endothelial localization of COX-2, the known vasodilatory effects of PGE2 (14), and the decrease in PGI2 after IL-1β treatment all support this hypothesis. As expected, the selective COX-2 inhibitor NS-398 had no effects on vascular tone of IL-1β-exposed arteries from estrogen-treated rats. However, in arteries from OVX rats, NS-398 partially reversed the decrease in tone caused by IL-1β. These results suggest the induction of additional vasodilators unrelated to COX-2. Therefore, signaling mechanisms downstream to the activation of COX-2 pathway activity because serum IL-1β levels were not affected by estrogen treatment. The expression of IL-1 receptors in cerebral vascular endothelium (15), the colocalization of COX-2 and IL-1 receptor mRNA in brain endothelial cells (4), and the rapid activation (20 min) of NF-κB demonstrated here all suggest that IL-1β is directly responsible for the induction of inflammatory prostaglandins in cerebral vessels. Therefore, signaling mechanisms downstream to the activation of IL-1 receptors may be possible targets for estrogen regulation.

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Fig. 8. Effects of estrogen treatment on NF-κB p65 DNA-binding activity after IL-1β treatment. With the use of an immunoassay-based kit, NF-κB p65 DNA-binding activity was measured in nuclear extracts from OVX and OVX+βE cerebral vessels isolated 20 min after injection of saline or IL-1β. Values are normalized to that of the positive control signal. Values are means ± SE; n = 3 or 4 rats; *P ≤ 0.05, significantly different.
suppresses NF-κB-dependent activation of COX-2 transcription. There is emerging evidence that the estrogen receptor complex, by utilizing transcriptional coactivators, can prevent NF-κB from inducing gene expression even when the pathway has been activated (45). The fact that 17α-estradiol, which is the inactive enantiomer of 17β-estradiol, did not suppress the induction of PGE2 by IL-1β supports the role of the estrogen receptor in mediating these effects.

The association of COX-2 products with the inflammatory sequelae of ischemic brain injury is well recognized. In fact, selective COX-2 inhibitors substantially reduce infarct volume (35) and neurological deficits (49) after middle cerebral artery occlusion in rodents. Reduction of PGE2 accumulation in the infarcted brain of animals treated with COX-2 inhibitors has also been noted (35). Furthermore, COX-2-null mice display less damage than wild-type mice after experimental stroke (25). Together these observations suggest that inflammatory prostaglandins contribute to ischemic brain injury, and suppression of COX-2 function may confer protection against stroke. Vascular-derived prostanooids in particular may play an important part in cerebral inflammation after ischemic stroke as they may exert a driving role in the initiation of the inflammatory cascade. For example, COX-2 is induced in cerebral vascular tissue after injury (13). COX-2 is primarily associated with production of PGE2 (22), although it can synthesize PG12 as well (1). The contribution of PGE2 to the inflammatory cascade is underscored by its ability to upregulate cytokine expression (21) and induce ICAM-1 expression in cerebral endothelial cells (47). Furthermore, the COX inhibitor indomethacin abrogates IL-1β and ischemia-mediated induction of VCAM-1, ICAM-1, and E-selectin in cultured cerebral endothelial cells (47). These findings suggest that PGE2 may indirectly influence monocyte and polymorphonuclear leukocyte adhesion and transmigration out of the vasculature and into the central nervous system, which is a process believed to contribute to secondary injury after acute experimental stroke (16). Therefore, suppression of inflammatory prostaglandin production in the vasculature may protect the brain after injury.

We have demonstrated that chronic in vivo 17β-estradiol treatment of ovarietomized rats almost completely inhibits the induction of cerebrovascular COX-2 and synthesis of PGE2 when assessed 3 h after IL-1β injection. This observation has also been reported in microglia (52) and uterine fibroblasts (42). Whether COX-2 and PGE2 are essential to the expression in the cerebral endothelium of cell adhesion molecules and whether this process is regulated by 17β-estradiol remains to be elucidated. Interestingly, 17β-estradiol suppresses cell adhesion molecule expression in cultured endothelial cells treated with cytokines (6, 17) and reduces leukocyte accumulation in cerebral venules in vivo (41).

In conclusion, systemic IL-1β induced NF-κB-dependent expression of COX-2 in the ovariectomized rat cerebral circulation, and COX-2 appeared to localize to the endothelial cell layer. Chronic 17β-estradiol treatment, however, inhibited IL-1β induction of COX-2 that was mediated in part by attenuation of NF-κB activation. As a result, estrogen prevented the IL-1β-induced upregulation of PGE2 in an estrogen receptor-dependent manner regardless of whether MPA was present. In addition, 17β-estradiol prevented the dramatic decrease in arterial tone that was observed after IL-1β exposure; however, suppression of COX-2 vasodilator pathways appeared to be only partially responsible for this effect. Although IL-1β and COX-2 pathways have been shown to contribute to ischemic brain injury, it remains to be determined whether suppression of cerebrovascular inflammatory prostanooid production can be added to the growing list of mechanisms by which estrogen acts as a neuroprotectant.

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REFERENCES