

## Soluble epoxide hydrolase: regulation by estrogen and role in the inflammatory response to cerebral ischemia

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### 1. ABSTRACT

The protection from ischemic brain injury enjoyed by females is linked to the female sex hormone 17 $\beta$ -estradiol. We tested the hypothesis that neuroprotection by estradiol entails the prevention of ischemia-induced inflammatory response, through suppression of the P450 eicosanoids-metabolizing enzyme soluble epoxide hydrolase (sEH). Ovariectomized female rats with and without estradiol replacement underwent 2-hour middle cerebral artery occlusion (MCAO). sEH expression was determined using Western blot, and inflammatory cytokine mRNA levels were measured at 6, 24 and 48 hours after MCAO. Cytokine mRNA was also measured in sEH-knockout mice, and in rats treated with sEH inhibitors. Estradiol reduced basal and post-ischemic sEH expression. MCAO strongly induced mRNA levels of tumor necrosis factor- $\alpha$ , interleukin 6, and interleukin 1 $\beta$ , which was attenuated in sEH-knockouts, but not by sEH inhibitors. Estradiol replacement exhibited a bimodal effect on cytokine mRNA, with increased early and reduced delayed expression. While estradiol suppresses cerebral sEH expression, and sEH suppression diminishes inflammation after MCAO, our findings suggest that the effect of estrogen on inflammation is complex, and only partially explained by sEH suppression.

### 2. INTRODUCTION

We previously demonstrated that female rats sustain smaller infarcts after middle cerebral artery occlusion (MCAO) compared to male rats (1), and that chronic estrogen replacement to physiological plasma levels is protective against ischemic brain injury in ovariectomized (2) and reproductively senescent (3) female rats. The mechanism of protection by estrogen is likely multifactorial involving vascular and non-vascular effects through genomic (4) and non-genomic (5) mechanisms. In the current study, we determined if estradiol's anti-inflammatory effect is mediated through suppression of soluble epoxide hydrolase (sEH), an enzyme that has previously been linked to inflammation (6) as well as stroke susceptibility (7) and outcome (8). Inhibition of sEH decreases lipopolysaccharide as well as tobacco-smoke induced inflammation (6, 9), and is neuroprotective in experimental stroke (10). Renal and hepatic expression of soluble epoxide hydrolase protein is lower in females than in males (11, 12), and sEH enzyme activity is reduced by estrogen and increased by testosterone treatment (13, 14), suggesting that sEH is regulated by sex hormones. Estrogen has been demonstrated to suppress inflammation in experimental models of multiple sclerosis (15, 16), traumatic brain injury (17) and cerebrovascular disease (18-

20). However, the effect of estrogen on postischemic cerebral inflammation (21, 22) is unclear. We therefore determined in the current study if estrogen replacement downregulates sEH expression in brain, whether inhibition of sEH suppresses the induction of inflammatory cytokines in brain after middle cerebral artery occlusion (MCAO), and whether estrogen replacement thereby diminishes ischemia-induced inflammatory cytokine expression in brain.

### 3. MATERIALS AND METHODS

The study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research, and protocols were approved by the Animal Care and Use Committee of Oregon Health and Science University. Rabbit polyclonal antibody against sEH as well as the sEH inhibitor 12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester (AUDA-BE) were kindly provided by Dr. Bruce Hammock, University of California, Davis. 4-phenylchalcone oxide (4-PCO) was purchased from BIOMOL (Plymouth Meeting, PA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

#### 3.1. Middle cerebral artery occlusion in rats

Female Wistar rats (Harlan, total  $n=97$ ) were ovariectomized (OVX) at the age of 10 to 12 weeks and allowed to recover for 7 days. A subgroup of rats ( $n=38$ ) was implanted subcutaneously with pellets containing 17 $\beta$ -estradiol (E2, 25 micrograms, 21-day controlled time release) 7 days before middle cerebral artery occlusion (MCAO), as previously described (2). Two-hour MCAO was induced using the intraluminal filament technique, as previously described (1). Briefly, animals were anesthetized with halothane (1% by mask in O<sub>2</sub>-enriched air), arterial blood pressure and gases were monitored through a femoral catheter, and rectal and head temperatures were monitored and controlled at normal values by heating lamps and warming blankets. Laser-Doppler cerebral cortical perfusion was monitored during vascular occlusion and the first 30 minutes of reperfusion. At 6, 24, or 48 hours of reperfusion, animals were re-anesthetized, blood was sampled by cardiac puncture for plasma estradiol measurement, and brains were quickly frozen in 2-methylbutane at  $-30^{\circ}\text{C}$  and stored at  $-80^{\circ}\text{C}$ . To test the effect of pharmacological inhibition of sEH on expression of inflammatory cytokines, rats were treated with sEH inhibitors 4-phenylchalcone oxide (4-PCO, 5 mg/kg i.p. at 30 min prior to MCAO) or 12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester (AUDA-BE, 10 mg/kg, i.p. at reperfusion), or with respective vehicles (dimethyl sulfoxide (DMSO) for 4-PCO and sesame oil for AUDA-BE). Baseline sEH expression and control cytokine levels were determined in naïve brains from E2-treated and untreated OVX rats ( $n=8$  each), and the effects of anesthesia and surgical stress were evaluated in sham-operated OVX and E2-treated rats ( $n=3$  each).

#### 3.2. Middle cerebral artery occlusion in mice

We used a mouse model of MCAO to determine the effect of sEH gene deletion on cytokine expression after

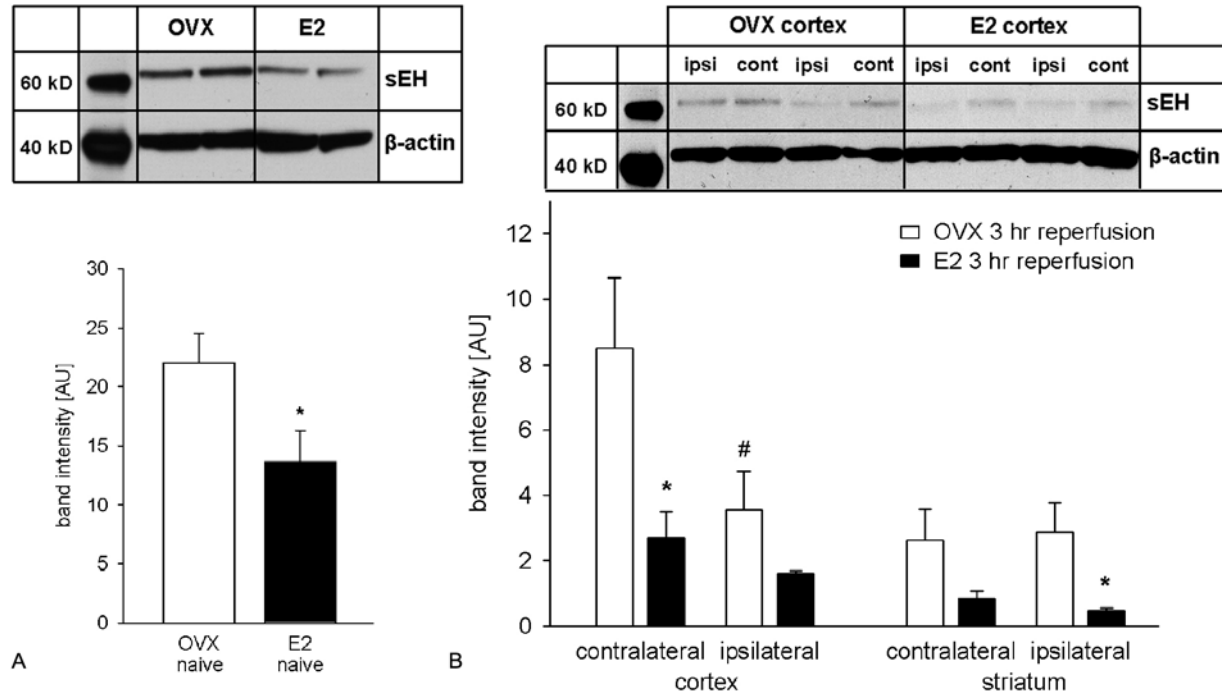
stroke. Male homozygous sEH-knockout mice (sEHKO, in-house colony originally obtained from Dr. Frank Gonzalez at the National Institutes of Health (23)) and age-matched C57/Bl6 wild-type (WT) controls (18–25 g, 10 weeks of age) were subjected to intraluminal MCAO, as previously described (24, 25). Briefly, under halothane anesthesia (1.5% in oxygen-enriched air by face mask), a 6-0 silicone-coated nylon monofilament was introduced through incision in the external carotid artery and advanced into the internal carotid artery to block flow through the MCA. A drop in laser-Doppler cerebrocortical perfusion to less than 20% of baseline indicated successful occlusion. Body temperature was monitored with a rectal probe, and normothermia was maintained throughout the procedure by using heated water pads. Mice were awakened after filament insertion, and evaluated at 2 hours for neurological deficit using a simple neurological scoring system as follows: 0=no deficit, 1=failure to extend forelimb, 2=circling, 3=unilateral weakness, 4=no spontaneous motor activity. Mice with clear neurological deficit were re-anesthetized for filament withdrawal to allow reperfusion as indicated by the return of Laser-Doppler perfusion to baseline. At 3 hours ( $n=7$  per genotype) and 24 hours ( $n=4$  per genotype) of reperfusion, brains were harvested and quickly frozen for RNA extraction and cytokine expression analysis. Naïve, non-ischemic mice ( $n=3$  per genotype) served as controls.

#### 3.3. Immunoblotting

For sEH expression analysis, the ipsilateral and contralateral cortex and striatum from the MCA territory were dissected and homogenized in solution A containing 250 mmol/l sucrose, 60 mmol/l potassium chloride, 15 mmol/l TrisHCl, 15 mmol/l sodium chloride, 5 mmol/l ethylenediaminetetraacetic acid (EDTA), 1 mmol/l ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.5 mmol/l phenylmethanesulphonylfluoride (PMSF), and 10 mmol/l dithiothreitol (DTT). Protein samples (15 micrograms) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, 90 min, 200 V) and transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen, Carlsbad, CA) at 35 V for 2 hours. After blocking in 5% dry milk phosphate-buffered saline containing 0.1% Tween (PBS-T), soluble epoxide hydrolase was detected using a primary rabbit antibody and a biotinylated secondary antibody (Amersham, GE Healthcare, Piscataway, NJ) with an ECL plus (Amersham) enhanced chemiluminescence detection kit. The blots were re-probed for beta-actin (Sigma) to ensure equal loading (26). Exposed X-ray films were scanned and analyzed using QuantityOne® (BioRad) software.

#### 3.4. RNase protection assay (RPA)

Total RNA was extracted from the ipsilateral and contralateral cerebral cortex and striatum between coronal levels +2 mm to  $-4$  mm relative to bregma using RNA STAT-60™ reagent (Tel-Test, Friendswood, TX), according to the manufacturer's instructions. This area corresponds to the core of the MCA territory in rat brain. In mice, RNA was isolated from the entire ipsilateral and contralateral hemispheres. RNA purity and integrity were assessed using denaturing agarose gel electrophoresis after



**Figure 1.** Estrogen and ischemia reduce sEH protein expression in brain: Soluble epoxide hydrolase (sEH) immunoreactivity is strong in brain tissue from ovariectomized (OVX) rats, but significantly reduced by estradiol replacement (E2) (1A, representative Western blot and summary graph,  $n=4$ ). After 2 hours of transient middle artery occlusion, sEH protein immunoreactivity is reduced in the ipsilateral (ipsi), compared to the contralateral (cont) cortex of OVX rats. This decrease in sEH protein is further augmented in both hemispheres by estrogen replacement (representative Western blot and summary graph in 1B,  $n=3$ ). \* $P<0.05$  vs. OVX, # $P<0.05$  vs. contralateral.

spectrophotometric quantification. Multi-probe RNase protection assay (Multi-Probe Template Set, rCK-1 for rat and mCK-2b and mCK-3b for mouse, BD RiboQuant<sup>TM</sup>, BD Biosciences, San Jose, CA) was used to simultaneously measure steady-state levels of the following cytokines: interleukins (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12, and IL-18, interleukin 1 receptor antagonist (IL1ra), tumor necrosis factor alpha and beta (TNF- $\alpha$ , TNF- $\beta$ ), lymphotoxin beta (LT- $\beta$ ), transforming growth factor beta (TGF- $\beta$ 1, TGF- $\beta$ 2, TGF- $\beta$ 3), macrophage migration inhibitory factor (MIF) and interferon beta and gamma (IFN- $\beta$ , IFN- $\gamma$ ), as well as the two housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and L32. High-specific-activity antisense riboprobes ( $\alpha^{32}$ P-labeled) were transcribed using the T7 RNA polymerase, as previously described (27). Samples (35 micrograms for rat and 25 micrograms for mouse) were incubated with pre-labeled probes, digested with RNase and resolved via electrophoresis on a denaturing polyacrylamide gel. Protected fragments were visualized on a PhosphorImager (Bio-Rad FX, Bio-Rad, Hercules, CA), and optical density was measured using QuantityOne<sup>®</sup> software (Bio-Rad) and normalized to L32 housekeeping gene.

### 3.5. Statistics

Data are presented as mean  $\pm$  standard error of the mean (SEM). Two-way ANOVA followed by posthoc Neuman-Keul analysis was used to compare cytokine

mRNA expression over time in OVX and E2 rats. Differences in protein levels between estradiol-treated and untreated animals, cytokine mRNA levels between sEH-knockout and wild-type mice, estrogen levels, and physiologic parameters were analyzed with Student's t-test. Statistical significance was set at  $p<0.05$ . Analysis was performed using SigmaStat software (SPSS, Chicago, IL).

## 4. RESULTS

### 4.1. Estrogen and ischemia reduce sEH protein expression in brain

We used an estradiol-replacement regimen known to reduce infarct size in our transient MCAO model to determine the effect of estradiol on brain sEH protein expression. Immunoblotting with anti-sEH antibody consistently detected strong immunoreactivity for sEH in brain tissue from ovariectomized rats (Figure 1A). In animals receiving estradiol-replacement, however, the amount of sEH in brain was significantly reduced (40% reduction, from  $22.1 \pm 2.4$  to  $13.7 \pm 2.7$  arbitrary units [AU],  $n=4$  per group, respectively,  $p<0.05$ ). Moreover, we also observed a reduction in sEH protein in ipsilateral, ischemic cortex compared to contralateral cortex starting at 3 hours after transient MCAO ( $3.6 \pm 1.2$  vs  $8.5 \pm 2.1$  AU, Figure 1B,  $n=3$  per group). Estrogen replacement further decreased the levels of sEH protein in ischemic animals in both hemispheres, compared to OVX (Figure 1B).

#### 4.2. Deletion of sEH gene suppresses ischemia-induced cytokine expression

To determine if sEH suppression may be beneficial in cerebral ischemia by altering the brain's inflammatory response, we compared inflammatory cytokine expression in brains from wild-type and from sEH-knockout mice after MCAO. Cytokine mRNA levels were low in non-ischemic control mouse brain. Expression levels of tumor necrosis factor alpha (TNF-alpha), interleukin 6 (IL-6), interferon gamma (IFN-gamma), interleukin 1 alpha (IL-1alpha), and interleukin 1 beta (IL-1beta) mRNA were strongly increased in the ipsilateral hemisphere in wild-type animals 3 and 24 hours after MCAO, as compared to levels in control WT brain (26.6 $\pm$ 6.4, 20.6 $\pm$ 2.8, 3.1 $\pm$ 0.4, 1.9 $\pm$ 0.3, 8.0 $\pm$ 1.8-fold induction at 3 hours, 35.7 $\pm$ 11.3, 28.9 $\pm$ 15.9, 7.2 $\pm$ 3.2, 1.4 $\pm$ 0.1, 7.5 $\pm$ 3.2-fold at 24 hours, respectively; Figure 2A and 2B). The induction of TNF-alpha, IL-6, IFN-gamma, and IL-1beta was significantly attenuated in brains of sEH-knockout compared to wild-type animals at 3 hours of reperfusion ( $p < 0.05$ ).

We also determined the effect of pharmacological inhibition of sEH on cytokine mRNA expression after MCAO. We used two mechanistically different inhibitors, 4-PCO and AUDA-BE, both of which reduce infarct size after MCAO in rats and mice (10). Surprisingly, neither inhibitor affected the level of cytokine mRNA expression in brain after MCAO. Cortical and striatal cytokine mRNA levels after MCAO were not different between animals treated with 4-PCO or AUDA-BE compared to animals treated with the respective vehicle (Figure 2C and Figure 2D).

#### 4.3. Effect of estrogen on ischemia-induced cytokine mRNA expression in brain

To evaluate the effect of estradiol replacement on cytokine expression in brain, we compared cytokine mRNA expression in OVX and E2-treated rats after MCAO. Cytokine levels were low in control (naïve) and sham-operated animals. Four inflammatory cytokines were consistently detected by RNase protection assay in all samples: TNF-alpha, IL-6, IL-1alpha, and IL-1beta. Starting at 6 hours after MCAO, the mRNA levels of TNF-alpha, IL-6 and IL-1beta were strongly induced in the ipsilateral, ischemic cortex (7.1 $\pm$ 1.9, 85.1 $\pm$ 37.6 and 10.8 $\pm$ 5.0 folds relative to naïve control,  $n = 8$  per group, respectively, Figure 3B) and striatum of OVX animals (4.3 $\pm$ 0.7, 41.2 $\pm$ 11.8 and 4.3 $\pm$ 1.3 folds,  $n = 8$ , respectively, Figure 3C). Levels of TNF-alpha and IL-6 mRNA reached a peak at 24 hours (5.3 $\pm$ 0.8 and 6.9 $\pm$ 0.9 folds for TNF-alpha, and 117.8 $\pm$ 66.0 and 62.6  $\pm$ 24.3 folds in cortex and striatum,  $n = 8$ , respectively), and decreased at 48 hours after MCAO. In contrast, the level of IL-1beta mRNA remained elevated (6.8 $\pm$ 2.2 and 4.2 $\pm$ 0.7 folds in cortex and striatum at 48 hours,  $n = 8$ ). The level of IL-1alpha mRNA was very low in brain, and transiently increased in the cortex only at 6 hours after MCAO (Figure 3).

The induction of TNF-alpha, IL-6, IL-1beta and IL-1alpha in ipsilateral striatum was significantly

suppressed 24 hours after MCAO in estradiol-replaced compared to untreated OVX rats (Figure 3D and 3E,  $n = 8$ ,  $p < 0.05$ ). Early after MCAO, however, cytokine mRNA expression was augmented in the estradiol-replaced group. Levels of cytokine mRNA were significantly higher in estradiol-replaced compared to untreated OVX animals at 6 hours after MCAO (Figure 3D and 3E,  $n = 8$ ,  $p < 0.05$ ). No differences in cytokine mRNA levels were observed in the ipsilateral cerebral cortex at any time after MCAO between estradiol-treated and untreated groups ( $n = 8$  per group, data not shown).

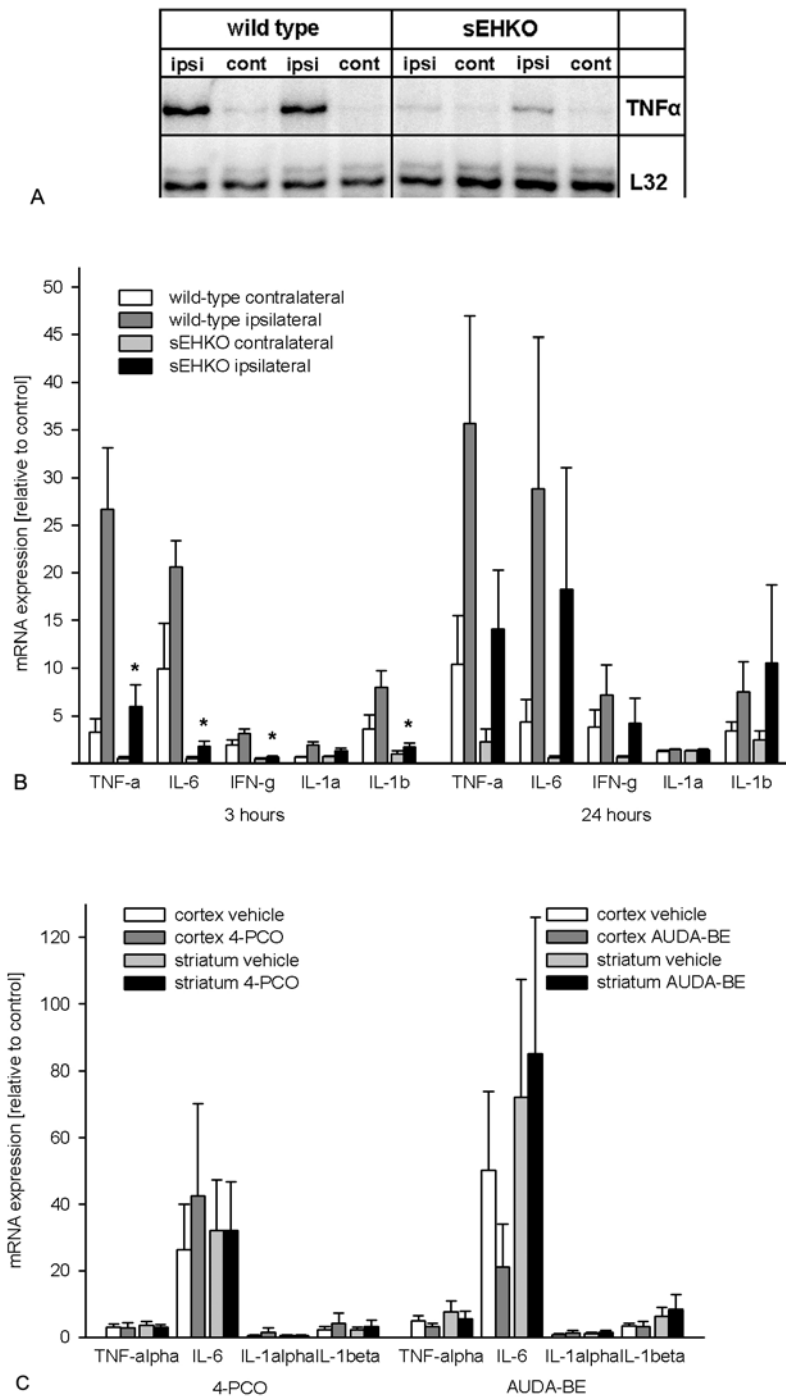
### 5. DISCUSSION

The main findings of our study are: 1) 17beta-estradiol (E2) replacement reduces baseline and post-ischemic expression of soluble epoxide hydrolase (sEH) protein in OVX rat brain, 2) gene deletion, but not pharmacological inhibition of sEH suppresses the ischemia-induced surge in cerebral inflammatory cytokine mRNA expression after MCAO, and 3) E2 replacement suppresses delayed, but enhances early induction of cytokine mRNA expression in brain after MCAO. We conclude that estrogen suppresses sEH in brain and modulates the brain's inflammatory response to ischemia. However, while sEH suppression attenuates inflammatory cytokine expression after MCAO, this does not fully explain the effect of E2 replacement on the inflammatory response to cerebral ischemia.

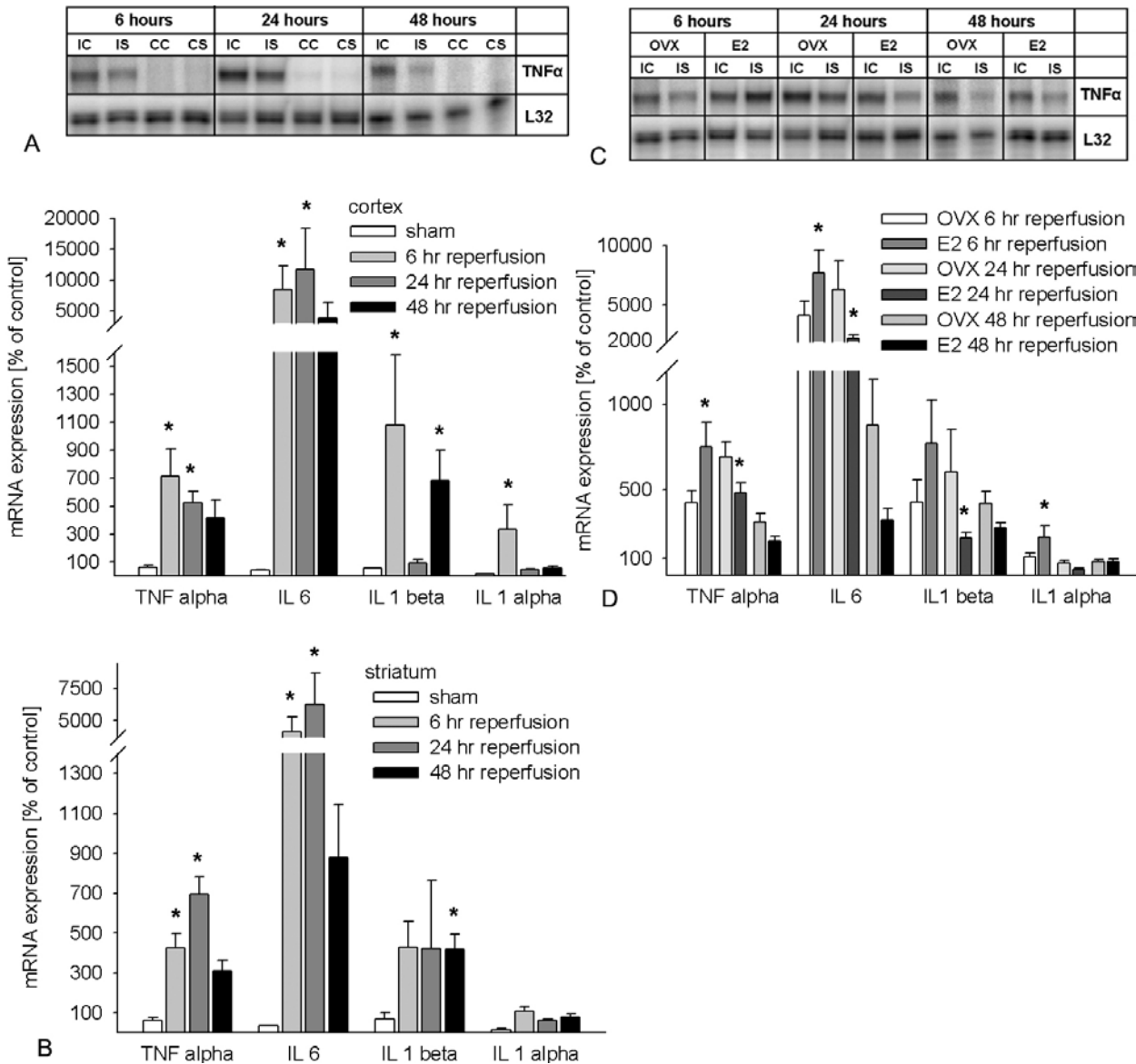
We have previously demonstrated that female rats sustained smaller infarcts after MCAO compared to age-matched males (1); however, the sex difference in infarct size disappeared after ovariectomy and was restored by estrogen replacement (2). The mechanism of protection by estrogen is multifactorial involving genomic (4) and non-genomic (5) mechanisms. In the current study, we tested the hypothesis that estrogen's neuroprotective property is in part mediated through anti-inflammatory mechanisms elaborated through estrogen's action to suppress sEH.

We show that sEH protein is abundantly expressed in OVX rat brain, and that its level of expression is significantly reduced by estrogen replacement. This is consistent with earlier studies demonstrating that sEH is expressed in liver and kidney in a sexually dimorphic manner, with higher levels in males compared to females (12-14). These studies also suggested that sEH levels might be regulated by sex hormones, since sEH levels were increased by ovariectomy and decreased by castration (13). Our study expands on these findings by demonstrating that 17beta-estradiol suppresses sEH protein expression in brain at baseline and after cerebral ischemia/reperfusion.

The effect of estrogen on sEH is consistent with an anti-inflammatory action. Pharmacological inhibition of sEH was shown to inhibit both systemic inflammation in response to lipopolysaccharide (6) as well as smoking-related lung inflammation (9). This anti-inflammatory effect of sEH inhibition is believed to be linked to increased bioavailability in brain of anti-inflammatory



**Figure 2.** Deletion of sEH gene, but not pharmacological inhibition, suppresses ischemia-induced cytokine expression: Transient middle cerebral artery occlusion increases cytokine mRNA levels in the ipsilateral hemisphere (ipsi) of wild-type (WT) mice as early as 3 hours after reperfusion (dark gray bars, left half of 2B). This increase is sustained at 24 hours of reperfusion (dark grey bars, right half of 2B). This cytokine mRNA induction is attenuated in sEH-knockout mice (sEHKO, black bars) 3 hours after reperfusion, and to a lesser degree at 24 hours. 2A shows a representative RNase protection assay illustrating increased ipsilateral TNF- $\alpha$  mRNA after middle cerebral artery occlusion in wild-type, but not in sEHKO mice; 2B summarizes data for  $n=8$  mice per group. Cont: contralateral hemisphere, \* $P<0.05$  vs. WT. Pharmacological inhibition of sEH with 4-phenylchalcone oxide (4-PCO, left half of 2C) or 12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester (AUDA-BE, right half of 2C) did not affect the induction of cytokine mRNA in ischemic cortex or striatum after middle cerebral artery occlusion.



**Figure 3.** Biphasic effect of estrogen on ischemia-induced cytokine expression: Transient middle cerebral artery occlusion induces mRNA of TNF- $\alpha$ , IL-6 and IL-1 $\alpha$  in the ipsilateral cortex (3B, upper panel) and striatum (3B, lower panel) of OVX rats, starting at 6 hours after ischemia (light gray bars). TNF- $\alpha$  and IL-6 mRNA reach a peak at 24 hours (dark gray bars), and decrease at 48 hours (black bars) after MCAO, whereas IL-1 $\beta$  remains elevated at 48 hours. White bars show mRNA levels in sham-operated animals. N=8, \*P<0.05 vs. sham. 3A shows representative RNase protection assay for TNF- $\alpha$  mRNA, illustrating increased mRNA expression after MCAO in ipsilateral cortex (IC) and striatum (IS), but not the contralateral side (CC, CS). Cytokine mRNA induction in ipsilateral striatum of OVX (3D) rats is significantly augmented by estradiol-replacement at 6 hours after MCAO (white and medium gray bars). At 24 hours, cytokine mRNA levels are lower in E2 compared to OVX animals (light gray and dark gray bars), whereas there is no difference between groups at 48 hours (gray and black bars). Representative RNase protection assay in 3C, summary of n=8 in 3D. \*P<0.05 vs. OVX.

eicosanoids referred to as epoxyeicosatrienoic acids (EETs) (28). EETs, which are preferentially metabolized by sEH, have been demonstrated to inhibit inflammation by suppressing endothelial expression of vascular cell adhesion molecule (VCAM-1) (29, 30) and preventing leukocyte adhesion (29). The relevance of the brain's inflammatory response to ischemic damage is well recognized: Ischemia/reperfusion injury elicits a strong

inflammatory reaction within the central nervous system. Inflammatory cytokines, initially released from ischemic cells, recruit leukocytes into the brain, which in turn produce more inflammatory mediators that exacerbate injury by activating microglia, disrupting the blood-brain barrier (BBB), inducing cell death and expanding tissue infarction. In support of a deleterious role of inflammatory mediators in cerebral ischemia, cytokine inactivation by

gene deletion or neutralizing antibodies decreased infarct size after MCAO in the rat (21, 31). In clinical studies, the level of inflammatory cytokines in serum or CSF of stroke victims correlated with long-term outcome (32-34), further emphasizing the detrimental role of cytokines in ischemic injury.

To determine if suppression of sEH inhibits the brain's inflammatory response to ischemia, we compared the levels of cytokine mRNA after cerebral ischemia between wild-type and sEH-knockout mouse brain. Cytokine expression after MCAO was significantly reduced in the brains of sEH-knockout mice compared to wild type controls, suggesting that EETs may play an anti-inflammatory role in brain following cerebral ischemia. In contrast, pharmacological inhibition of sEH using two structurally different agents failed to suppress the induction of cytokine mRNA in brain after cerebral ischemia in the rat. This was surprising since in our hands these two inhibitors, 4-PCO and AUDA-BE, reduce infarct size after MCAO (10). This was also puzzling since, as mentioned above, a previous study demonstrated that AUDA-BE abolished lipopolysaccharide-induced cytokine release systemically (6). The discrepancy between our finding and that of Schmelzer *et al* may be related to differences in the AUDA-BE dose used in both studies. The AUDA-BE dose required to suppress systemic inflammation was 20 mg/kg, which was administered twice, compared to a single injection of 10 mg/kg in our study (6). The choice of this dose and regimen in our study was based on our previous finding that a single 10 mg/kg AUDA-BE injection was sufficient to reduce infarct size after MCAO in mice (10). The fact that AUDA-BE reduces infarct size without suppressing early inflammatory cytokine expression after MCAO suggests that the mechanism of protection by AUDA-BE is not mediated through an anti-inflammatory mechanism of action. A possible explanation for the discrepancy in our study between the effects of sEH gene deletion compared to pharmacological inhibition may be related to the recent discovery that sEH is a bifunctional enzyme containing both phosphatase and hydrolase enzymatic activities (35, 36). While both of these enzyme activities are abolished by gene deletion (and reduced by E2-mediated gene suppression), only the hydrolase activity is inhibited by the pharmacological blockers, suggesting that while inhibition of the hydrolase conveys neuroprotection, suppression of the phosphatase may be required for anti-inflammation, at least in the brain.

To determine if E2-mediated suppression of sEH protein in brain is sufficient to reduce the inflammatory response to cerebral ischemia, we compared the levels of cytokine expression at different time points after MCAO between E2-replaced and untreated ovariectomized animals. We found a significant decrease in the mRNA expression of TNF-alpha, IL-6 and IL-1beta in estrogen-treated OVX rats at 24 hours after MCAO, consistent with an anti-inflammatory action of E2 at this time point. Surprisingly, cytokine levels at 6 hours after MCAO were higher in E2-treated compared to untreated OVX rats. This novel, albeit surprising finding that estrogen exhibits differential effects on early vs. delayed cytokine expression suggests that inflammatory cytokines may play differential

roles during early and delayed stages of reperfusion after cerebral ischemia. Immune cells, such as leukocytes, start infiltrating ischemic brain tissue between 12 and 24 hours of reperfusion (37, 38). Therefore, our observation that estrogen reduces delayed cytokine expression is consistent with previous reports demonstrating that estrogen replacement reduces leukocyte adhesion to cerebral vascular endothelium, an early stage of leukocyte infiltration, after forebrain ischemia (39). Reduced expression of cytokines in estrogen treated rat brains at 24 hours of reperfusion may be related to reduced leukocyte invasion into the ischemic tissue. While delayed expression and release of cytokines is likely to be mediated by leukocytes, and therefore serve a detrimental role in the pathogenesis of cerebral ischemia and the evolution of infarction, the early release of cytokines is unlikely produced by peripheral immune cells, but rather by parenchymal brain cells, such as neurons and astrocytes (40, 41). Cytokines released by neurons and astrocytes during the early hours of reperfusion may act as neurotrophic factors and serve a beneficial role in injured brain. For example, blockade of endogenous IL-6 has been shown to exacerbate neuronal apoptosis and increases infarct size after cerebral ischemia (42, 43), and TNF-alpha (44) and IL-1 (45) have been implicated in the induction of tolerance after ischemic preconditioning. Estrogen enhancement of cytokine expression at 6 hours after MCAO, as we have observed in this study, may therefore serve a beneficial role.

In summary, we report that estrogen suppresses sEH expression in brain, which may attenuate the inflammatory response to ischemia. We also report for the first time that estrogen modulates early and delayed cytokine expression in ischemic brain in a differential manner.

## 6. ACKNOWLEDGMENT

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**Abbreviations:** sEH: soluble epoxide hydrolase, OVX: ovariectomized, E2: estradiol, MCAO: middle cerebral artery occlusion, 4-PCO: 4-phenylchalcone oxide, AUDA-BE: 12-(3-adamantan-1-yl-ureido)-dodecanoic acid butyl ester, DMSO: dimethyl sulfoxide, mRNA: messenger ribonucleic acid, TNF: tumor necrosis factor, IL: interleukin, IFN: interferon, EETs: epoxyeicosatrienoic acids

**Key Words.** Inflammatory cytokines, cerebral ischemia, estrogen, neuroprotection, soluble epoxide hydrolase, epoxigenase, EETs, epoxigenase, eicosanoids, interleukins, TNF-alpha

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