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Mechanism of the Sex Difference in Neuronal Ischemic Cell Death

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Abstract

Background—Stroke risk and outcome are different in men and women. We hypothesized that this is partly due to an inherent difference in susceptibility to ischemia between neurons from male vs. female brains. We tested whether neurons from male rodents are more susceptible to in-vitro ischemia than cells from females, and if this is related to increased expression of soluble epoxide hydrolase (sEH). SEH contributes to neuronal cell death by inactivating neuroprotective epoxyeicosatrienoic acids (EETs).

Methods—Rodent cortical neurons were cultured, and exposed to oxygen-glucose deprivation (OGD); then cell death was measured. EETs levels were determined by LC-MS/MS. Expression of sEH-encoding ephx2 was determined by qRT-PCR. Western blotting, immunocytochemistry, and hydrolase activity assay assessed protein expression and activity.

Results—Cell death after OGD was higher in neurons from males vs. females, which correlated with higher ephx2 mRNA and stronger sEH immunoreactivity. However, EETs levels were similar in both sexes and pharmacological inhibition of the hydrolase domain of sEH did not abolish the sex difference in cell death. Genetic knockout of sEH in mice abolished the sex difference observed in neurons isolated from these mice after OGD.

Conclusions—Cultured cortical neurons from females are more resistant to ischemia than neurons from males. Neurons from females have less sEH activity compared to neurons from males at baseline, although sEH levels were not measured after OGD. While pharmacological inhibition of the hydrolase domain of sEH does not affect cell death, knockout of the gene encoding sEH eradicates the sex difference seen in wild-type neurons, suggesting a role for further study of the lesser-known phosphatase domain of sEH and its role in sexual dimorphism in neuronal sensitivity to ischemia.

Keywords

Acute Stroke; EETs; Brain Ischemia; Gender; Soluble epoxide hydrolase

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Introduction

Important differences in stroke risk and outcome exist between men and women. In general, stroke incidence and mortality rates are lower in premenopausal women relative to men of the same age (Reeves et al. 2008). The female advantage in relation to stroke risk is present in childhood (Golomb et al. 2008) and persists after menopause (Lloyd-Jones et al. 2009), suggesting that some of the sex differences are unrelated to sex hormones and are, in fact, inherent gender differences at the cellular level. Evidence for inherent genetic differences between males and females have been documented recently in cellular sub-populations in the brain, in neurons in particular. For example, studies conducted using cultured neurons in the absence of sex hormones have shown that cell viability is, in part, dependent on whether cells are derived from the male or female brain (Lei Zhang et al. 2003). In addition, sex differences in neuronal survival have been observed in response to cytotoxic and apoptotic stimuli (Lieb et al. 1995) (Du et al. 2004). These observations suggest that male and female neurons may have inherently different susceptibility to ischemia, and that this difference is triggered by innate variations between the sexes in gene regulation and protein expression that are independent of post-natal exposure to sex hormones.

A potential gene involved in the sexual dimorphism of neuronal survival is EPHX2, the gene coding for the protein soluble epoxide hydrolase. Soluble epoxide hydrolase (sEH) is a protein known to be sexually dimorphic in the whole brain, and in liver and kidney, but it is not known if it is sexually dimorphic in neurons, and if it mediates the sex difference in neuronal ischemic sensitivity. sEH is a heterodimer that possesses a C-terminal hydrolase domain as well as an N-terminal phosphatase domain. The C-terminal hydrolase metabolizes and inactivates a lipid signaling molecule called epoxyeicosatrienoic acids (EETs) via hydrolysis. EETs have been shown to protect neurons from ischemic injury both in vivo and in vitro(Iliff and Nabil J Alkayed 2009), and sEH inhibition and gene deletion have also been shown to be protective against ischemic injury(Wenri Zhang et al. 2009)(W Zhang et al. 2008). The N-terminal phosphatase domain of sEH is less studied but may be involved in fatty acid metabolism (Newman et al. 2003) and may participate in the regulation of eNOS activity in vivo (Hou et al. 2011).

In the current study, we sought to explore the mechanism of sexual dimorphism in neuronal ischemic sensitivity at the cellular level. Using an in vitro model of ischemia, oxygen-glucose deprivation (OGD), we examined cell death in relation to sEH expression and activity in neurons derived from male versus female murine fetus. Additionally, we measured total intracellular EETs levels in these cultures using LC-MS/MS. Finally, we examined the effect of pharmacological and genetic ablation of sEH on cell death.

Materials and Methods

This study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research and the protocols were approved by the Animal Care and Use Committee of Oregon Health & Science University.

Neuronal Cell Culture

All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) except as specified. Highly-enriched neuronal cultures were prepared from embryonic day 18 Sprague-Dawley rat fetuses (Charles River, Wilmington, MA) as previously described (I P Koerner et al. 2007) or embryonic day 16 mouse fetuses from C57BL/6 (Charles River) or sEH knockout (sEHKO) mice as previously described (Jia et al. 2011). Rat and mouse fetuses were separated by sex after laparotomy and visual inspection of internal sex organs.

Rat Neuronal Culture

Briefly, brains were removed; cortices were dissected in HEPES-buffered HBSS and dissociated by digestion with trypsin and trituration. Neurons from male (N_M) and female (N_F) littermates were cultured side-by-side and were seeded at a density of 1.5×10^5 cells/cm^2 onto poly-D-lysine coated plates. Neurons were grown in Neurobasal medium without phenol red supplemented with 2% B27, 1% Glutamax, and 1% penicillin/streptomycin. Cytosine-1-β-D-arabino furanoside (Ara-C, 1 μM) was added to the culture medium on DIV 3 to suppress growth of glial cells. Cultures consisted of >98% microtubule-associated protein 2 (MAP2)-positive neurons and <2% glial fibrillary acidic protein (GFAP)-positive astrocytes.

Mouse Neuronal Culture

After determining sex, brains were removed; cortices were dissected in HEPES-buffered HBSS. Tissue was digested with a 0.5 mg/mL papain solution (Worthington Biochemical Corporation, Lakewood, NJ) at 37°C for 8 minutes. Papain was removed and tissue was washed twice with Trypsin Inhibitor (1 mg/mL, Trypsin Inhibitor, soybean, Sigma-Aldrich, St. Louis, MO) for 2 minutes. Cells were rinsed once with neurobasal medium and then dissociated into a total of 10 mL neurobasal medium via tituration with a 5 mL pipette. Cells were then spun at 1,000 rpm for 5 minutes, supernatant was removed; cells were resuspended in fresh neurobasal medium and filtered with a cell strainer. Cells were then counted and plated at equal densities as described above.

Soluble epoxide hydrolase knockout (sEHKO) mice

Mice with targeted deletion of sEH (sEH knockout/sEHKO) were used only for the sEHKO cell death experiments. The mice originated on a B6;129X1 background and have been backcrossed to C57BL/6 for more than 7 generations, as previously described (W Zhang et al. 2008). Homozygous sEHKO mice are viable, fertile, normal in size, and phenotypically identical to C57BL/6 mice. Mice were genotyped by PCR as previously described (W Zhang et al. 2008)(Sinal et al. 2000).

Oxygen-Glucose Deprivation

To simulate ischemia, cells were subjected to oxygen-glucose deprivation (OGD) on DIV 10. OGD was performed as published in Koerner, et al (2007) (I P Koerner et al. 2007). Briefly, culture plates were placed in an anaerobic chamber (COY Laboratory Products, Grass Lake, MI) filled with anoxic gas mixture (5% CO_2, 5% H_2, 90% N_2). Oxygen concentration was maintained at 0 parts per million (ppm) using a palladium catalyst. Culture medium was then replaced with Dulbecco's Modified Eagle Medium (DMEM) without glucose, and cells were maintained in the OGD chamber for 2 hours. After 2 hours of OGD, DMEM was exchanged with prewarmed culture growth medium, and cells were returned to the normoxic incubator for 24 hours. After 24 hours of reoxygenation, neuronal cell death was assessed as described below.

Drug Treatment

Primary cultured cortical neurons were treated with 2 μM 4-phenylchalcone oxide (4-PCO, Biomol, Plymouth Meeting, PA). Treatment was started one hour before OGD and continued throughout the OGD/reperfusion period. 4-PCO was dissolved in Dimethylsulfoxide (DMSO), and DMSO was used as vehicle control. The drug's concentration and treatment duration were the same as previously used in Koerner et al (Ines P Koerner et al. 2008).

Assessment of Cell Death

Cell death was determined by the release of lactate dehydrogenase into the media (LDH Cytotoxicity Detection Kit; Roche Diagnostics, Basel, Switzerland). Data from 3 to 5 wells per condition per experiment were averaged to n=1. Each experiment represents an independent culture from a separate litter. Cell death was confirmed by the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is converted by viable cells to a formazan that can be measured spectrophotometrically at 540 nm as an indicator of cell viability

Hydrolase Activity Assay

Soluble epoxide hydrolase activity was determined using Epoxyfluor 7 (EP7), (Cayman Chemical Company) as previously described (Jones et al. 2005). Cells were lysed in PBS on ice before immediate quantification of hydrolase activity. Reactions were carried out in 200 μL of 25 mM BisTris-HCl containing 1 mg/mL bovine serum albumin (BSA) and the substrate EP7 (5 μM). The resulting solution was incubated at 37°C for 60 minutes in a black 96-well flat bottom plate (Corning, Corning, NY). Fluorescence of hydrolyzed EP7 was determined using an excitation wavelength of 330 nm (bandwidth=20 nm) and an emission wavelength of 465 nm (bandwidth=20 nm) on a plate reader (VICTOR, Wallac / Perkin Elmer, Waltham, MA). Activity was normalized to sample protein concentration and expressed as relative fluorescence units (RFU). All determinations were performed with at least three replicates.

Immunocytochemistry

To localize sEH, a primary rabbit polyclonal antibody against sEH (1:1000, a gift from Dr. Bruce Hammock) was used. A cy-2 labeled goat secondary antibody was used for labeling (1:2500, Jackson ImmunoResearch).

Rat cortical neurons were plated onto 18 mm poly-D-lysine coated glass coverslips. On DIV 7, coverslips were washed, fixed with 4% paraformaldehyde in PBS for 10 min, washed and blocked with blocking buffer consisting of 5% normal goat serum (Jackson ImmunoResearch, West Grove, PA), 1% bovine serum albumin (BSA, Sigma), 0.2% Fish Gelatin, and 0.1% Triton-X (Sigma) in PBS. Coverslips were incubated with primary antibodies diluted in blocking buffer at 4°C overnight, washed with 0.1% Tween-20 in PBS (PBS-T), and incubated with secondary antibody for 2 hrs at room temperature. Finally, Hoechst 33342 (Invitrogen) stain was applied to the coverslips then rinsed once with PBS-T. Coverslips were then mounted with Southern Biotech Fluoromount-G mounting media (InterScience, Markham, ON, Canada).

Slides were photographed using a Zeiss 710 laser confocal microscope. Identical conditions were applied for each photograph. sEH immunofluorescence was quantified using Imaris software (BitPlane Scientific Software, Zurich, Switzerland). The sum of fluorescent pixels was obtained for three fields of view and averaged per slide and the sum of the background fluorescence was subtracted. Three slides each from at least four independent cell culture experiments were used for analysis (n=4 biological replicates).

Western Blot

Cells were lysed in a solution containing sucrose (250mM), potassium chloride (60 mM), tris(hydroxymethyl)aminomethane hydrochlorate (15mM), sodium chloride (15 mM), ethylenediaminetetraaacetate (5mM), ethylene glycol tetraacetic acid (1 mM), phenylmethanesulfonylfluoride (0.5mM) and dithiothreitol (10 mM). Cell lysates were then centrifuged at 2000× g for 10 minutes at 4°C. Protein samples (50 μg) were separated by gel electrophoresis and then transferred to Polyvinylidene Difluoride (PVDF) membranes. Blots

were blocked in 5% dry milk, and incubated at 4°C overnight with a primary rabbit polyclonal antibody against murine sEH (1:100, Cayman Chemical). The signal was visualized using a horseradish peroxidase-linked (HRP) rabbit secondary antibody (1:500, GE Healthcare, Piscataway, NJ) followed by detection using Supersignal chemiluminescent reagents (Pierce). Images were obtained on a Fluorchem FC2 MultiImage II (Alpha Innotech, St. Leandro, CA) and band optical densities were quantified and expressed relative to GAPDH.

TaqMan Real-Time Quantitative RT-PCR

To quantify *ephx2* mRNA expression in cultured cells, total RNA was extracted using a RNAqueous-Micro kit (Ambion, Foster City, CA). First-strand cDNA was prepared using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). TaqMan® quantitative PCR reactions were performed in a 96-well plate on an ABI Prism 7000 DNA Detection System. The following specific probe and primer sets for ephx2 were designed with Primer Express software (Applied Biosystems): 5'-FAM-cca gcc cag tca tgg cca at-TAMRA-3 (Probe); 5'-act ggg aat ccc tca agca-3' (Forward Primer); 5'-aga gag cca tat tcc aca ccag-3' (Reverse Primer). Each sample was run in triplicate. Levels of *ephx2* were normalized to 18S RNA (18s rRNA Control Kit, Eurogentec. Fremont, CA).

Liquid Chromatography-Tandem Mass Spectrometry

EETs quantification in primary cultured cortical neurons was determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Primary cultured cortical neurons were grown in 6-well cell culture dishes for 10 days. The cells were exposed to OGD on DIV 10 and compared to sham controls. After 24 hours of reoxygenation, cells were washed with PBS, then scraped and collected. Methods are identical to previous published methods (Iliff et al. 2010), except that protein was quantified for each sample; therefore, absolute EETs concentrations are available.

Statistics

All values are expressed as the mean ± standard error of the mean (SEM). Statistically significant differences between groups were determined by a t-test for two groups or analysis of variance (ANOVA) for multiple groups using the Sigmastat software (Systat Software, Inc., Chicago, IL). When using ANOVA, two-way ANOVA of the difference of paired means (i.e. repeated measures) was used to allow for a paired littermate analysis (males vs. females of the same litter), comparing the factors of treatment group and sex. This analysis controlled for variability in absolute cell death values among cultures. The post-hoc Student Newman-Keuls tested for differences between treatment group and sex. $P<0.05$ was considered significant.

Results

Neurons from Female Rodents were Less Susceptible to Oxygen-Glucose Deprivation

When cell death was compared between primary cultured cortical neurons from male (N_M) vs. female (N_F) embryos of the same litter, N_F were consistently more resistant to OGD than N_M. This was true in both rat and mouse species. On average, as a percentage of cell death in corresponding N_M, N_F sustained 16.3±4.5% less death. These results are summarized in Figure 1, which compares cell death data from N_M and N_F cultured from the rat brain, showing that cell death in N_F was significantly less than cell death in N_M (n=9, $p<0.05$).

Sex Differences in Quantity and Activity of Soluble Epoxide Hydrolase in Neurons

In order to investigate a potential contributing mechanism for the sex differences exhibited in cell death after in vitro ischemia, we used several techniques to measure the expression and activity of sEH and EETs, which are known to be involved in neuroprotection(I P Koerner et al. 2007)(Wenri Zhang et al. 2009). We measured sEH C-terminal hydrolase activity because robust activity of the hydrolase would suggest that neuroprotective EETs levels would be reduced. Using the epoxyfluor 7 (EP7) assay to measure hydrolase activity, we found that it was indeed higher in primary cultured rat cortical N_M when compared to N_F (Figure 2, n=6, p=0.01). After determining that N_M exhibit significantly more hydrolase activity than N_F, we hypothesized that sEH protein expression would be higher in N_M compared to N_F. We investigated this using both immunocytochemistry and Western blotting with anti-sEH antibody. sEH immunoreactivity was detected in both rat N_M and N_F. Figure 3A represents confocal microscopy images showing increased expression of sEH. Figure 3B is a graphical representation of sEH fluorescence, demonstrating the increased expression of sEH (by 53%) in N_M compared with N_F (n=4, p=0.011). Additionally, Western blotting demonstrates that N_M had consistently more sEH compared to N_F littermates (Figure 4, n=4, p=0.004).

Sex Differences in the Level of EPHX2 mRNA Before and After OGD

We used quantitative real-time RT-PCR (qPCR) to determine the sex difference and effect of ischemia on EPHX2, the gene encoding sEH. At baseline and after 2 hrs OGD, rat N_M had significantly more EPHX2 mRNA when compared to N_F (Figure 5, n=6, p<0.05). Levels of EPHX2 were much more variable in both N_M and N_F after OGD. Further, OGD did not affect EPHX2 mRNA, since there was no significant difference between baseline and post-OGD levels of EPHX2 mRNA in either group.

14,15-EET was Present in Male and Female Cells at Baseline and After OGD

Because sex differences exist in both quantity and activity of sEH, we determined whether sex differences also exist in the quantity of 14,15-EET, which is synthesized from arachidonic acid by cytochrome P450 epoxygenase enzymes and hydrolyzed by sEH. Figure 6A depicts representative peaks used in the quantification of 14,15-EET in cells using LC-MS/MS. Interestingly, there was no significant difference in total 14,15-EET concentrations between rat N_M and N_F at baseline (Figure 6B, n=5, p=0.07). After OGD 14,15-EET significantly decreased in N_F, but not N_M (n=5, p=0.015). Other EETs regioisomers were also present, albeit at lower concentrations, and similar to 14,15-EET, there were no sex differences in other EETs (data not shown).

We also measured 14,15-EET concentration in media, to account for the possibility that EETs are released from the cells in response to OGD; however, the media EETs concentrations were highly variable and no conclusions could be made.

Inhibition of sEH Using 4-PCO

To determine if the sex difference in sEH contributes to the sex difference in ischemic cell death, we tested whether treatment with an sEH inhibitor would abolish the sexual dimorphism seen in neuronal ischemic cell death. We used 4-Phenylchalcone Oxide (4-PCO), a known inhibitor of sEH (Koerner *et al*, 2007). 4-PCO inhibits the hydrolase domain of sEH without inhibiting the lesser-known phosphatase domain(Newman et al. 2003). There is no known inhibitor currently available that specifically inhibits the phosphatase domain of sEH. We found that at a dose of 2 μM, 4-PCO inhibited 97% of the hydrolase activity of recombinant sEH when tested with the Epoxyfluor 7 hydrolase activity assay (data not shown). Accordingly, on DIV 10, primary neuronal cultures were treated with 2

μM 4-PCO or DMSO vehicle one hour prior to OGD, and treatment continued throughout OGD and 24 hours of reoxygenation. Cell death was lower in N_F vs. N_M neurons regardless of treatment, but 4-PCO did not eliminate the sex difference in cell death (Figure 7, n=7).

Neurons Cultured from Male and Female sEH Knockout Animals were not Differentially Susceptible to Ischemia

In order to further test whether sEH may be involved in the sexual dimorphism in neuronal cell death, we cultured neurons from sEH knockout (sEHKO) mice and exposed them to OGD. Contrary to our findings using the sEH hydrolase inhibitor 4-PCO, we found that total deletion of sEH completely abolished the sex difference in ischemic neuronal cell death. As shown in Figure 8, whereas WT neurons retain the sex difference in ischemic sensitivity (n=6–8, p=0.026), neurons derived from sEHKO mice did not demonstrate a sex difference in cell death after OGD.

Discussion

This study demonstrates four important findings. First, neurons derived from male and female murine cortices responded differently when exposed to OGD. Second, we, for the first time were able to detect sEH and endogenous 14,15-EET in primary cultured cortical neuronal cells. Third, the sex difference in susceptibility to OGD was associated with baseline differences in activity and expression of sEH, although sEH levels were not measured after OGD. Lastly, inhibition of the hydrolase activity of sEH did not eliminate the sex difference in cell death after in vitro ischemia, but cells from genetic knockout of sEH did eliminate the sex difference. Based on these findings, we conclude that ischemic neuronal cell death is sexually dimorphic, and the sex difference is linked to differences in sEH, which could not be explained by differences in the enzyme's hydrolase activity and levels of EETs.

Sex differences in stroke have been observed and reported using both in vitro and in vivo models of ischemic neuronal injury. However, this difference is usually attributed to the exposure to, and protection by, estrogen. For example, we previously demonstrated that adult female rats sustain smaller infarcts after experimental stroke induced by middle cerebral artery occlusion (MCAO) compared to age-matched males (N J Alkayed et al. 1998). The sex difference in infarct size is partly due to the protective effect of the estrogen, because absence of estrogen due to ovariectomy increases ischemic brain damage in female rats, and estrogen replacement is protective against cerebral ischemia in ovariectomized and reproductively senescent male and female rats (Rusa et al. 1999)(N J Alkayed et al. 2000). In our experiments, neuronal cells were grown in steroid-free media in the absence of sex hormones; therefore, the sex differences in neuronal cell death cannot be explained by differences in sex hormone exposure. Our experiments focused on the absence of sex hormones in culture, so organizational effects of the sex hormones on the developing embryo in utero until the day of culture cannot be completely discounted.

Soluble epoxide hydrolase is involved in the metabolism and terminal inactivation of EETs, which are lipid signaling molecules. We previously demonstrated that sEHKO male mice were protected from ischemic brain injury and that protection was associated with higher blood flow during MCAO compared with WT male mice (W Zhang et al. 2008). Soluble epoxide hydrolase is known to be sexually dimorphic in multiple tissues, including whole brain (Wenri Zhang et al. 2009), but whether it is sexually dimorphic specifically in pure neuronal cultures had not been investigated. Here we tested the hypothesis that N_F have lower sEH expression compared to N_M. Indeed, we were able to demonstrate that N_F have lower baseline sEH expression, lower hydrolase activity, and decreased levels of EPHX2 mRNA, although sEH levels were not measured after OGD. The physiologic role of sEH in

neurons is not completely understood. It is known that the C-terminal hydrolase domain of sEH is involved in metabolizing EETs. EETs protect against ischemic brain injury in vivo(Wenri Zhang et al. 2009) and against neuronal cell death induced in vitro by OGD (I P Koerner et al. 2007). This protection is by multiple mechanisms, including vasodilation, cytoprotection and suppression of post-ischemic inflammation (Iliff and Nabil J Alkayed 2009).

Because we found that N_M have increased sEH expression and activity compared with N_F, we hypothesized that the increase in sEH in N_M leads to decreased protective EETs levels, which, in turn would lead to increased susceptibility to ischemic cell death. Surprisingly, we did not see a corresponding difference in baseline levels of EETs between N_M and N_F. We did, however, find a difference in how male- and female-derived neurons respond to OGD. Specifically, N_F had significantly less total cellular EETs levels after OGD compared to baseline, whereas there is no change in N_M following OGD. EETs are incorporated into membrane phospholipids and released in response to phospholipase A2 (PLA2) activation (Bernstrom et al. 1992). Therefore, the decrease observed in N_F possibly represents a stronger PLA2 response to OGD. We could not confirm this hypothesis by measuring EET levels in media due to high variability, although the average concentration of 14,15-EET in media indeed tended to increase in N_F after OGD, while in N_M, no such trend was observed.

A possible explanation for the lack of difference in EETs levels between male- and female-derived neurons is that the sex difference may be linked to a different, non-eicosanoid substrate for the hydrolase activity of sEH. Contrary to what we had predicted, pharmacologic inhibition of sEH using 4-PCO did not abolish the sex difference in ischemic cell death in the neurons, despite 4-PCO being an effective inhibitor of the hydrolase activity in vitro. Interestingly, the sex difference in ischemic cell death was abolished when we used neurons cultured from sEH null mice (sEHKO). This finding suggests that, while the sex difference in *hydrolase* activity may not contribute to the sex difference in cell death, the secondary function of sEH, i.e., its *phosphatase* activity, may contribute to the difference in neuronal cell death. In support of this notion, in vivo studies have also observed a discrepancy between the effects of pharmacological sEH inhibition (which inhibits only hydrolase) versus total sEH gene deletion (which removes both hydrolase and phosphatase). For example, sEH gene deletion in the sEHKO mouse reduces inflammatory cytokine expression in brain after stroke, whereas pharmacological inhibition did not have any effect on cerebral inflammation after stroke (Ines P Koerner et al. 2008). Similarly, sEH gene deletion was associated with increased blood flow after MCAO in the mouse (W Zhang et al. 2008), while pharmacological inhibition was not (W Zhang et al. 2008). These observations suggest that the enzymes hydrolase activity does not fully account for all phenotypes observed by complete sEH deletion. This may be related to unrecognized roles for the phosphatase domain, a domain that remains largely unstudied due to lack of specific tools to evaluate it and because its endogenous substrate, if any, remains unidentified. Limitations to these observations include the validity of comparing data from a pharmacological inhibitor with data from a genetic knockout. The sEHKO mice are completely devoid of sEH throughout the animal's entire life, allowing for possible developmental compensations that may influence study outcomes. Use of conditional knockouts in future experiments would help to further clarify this issue.

In summary, primary cultured cortical neurons exhibit an innate sex difference in response to ischemic cell death, with cells from female murine brain exhibiting less death when compared to male-derived neurons. The mechanism supporting this cell difference is yet to be determined, but may involve the phosphatase domain of sEH. Elucidating innate sex-specific mechanisms of brain injury after stroke may allow the development of sex-tailored and more effective therapies against stroke injury in both men and women.

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Highlights

1. We examine the effects of in vitro ischemia on cultured neurons.
2. Cultured neurons from female brain are more resistant to in vitro ischemia than neurons from males.
3. Neurons from females have less sEH expression than those from males at baseline.
4. Levels of sEH were not measured after OGD.
5. Knockout of the gene encoding sEH eradicates the sex difference in wild-type neurons.

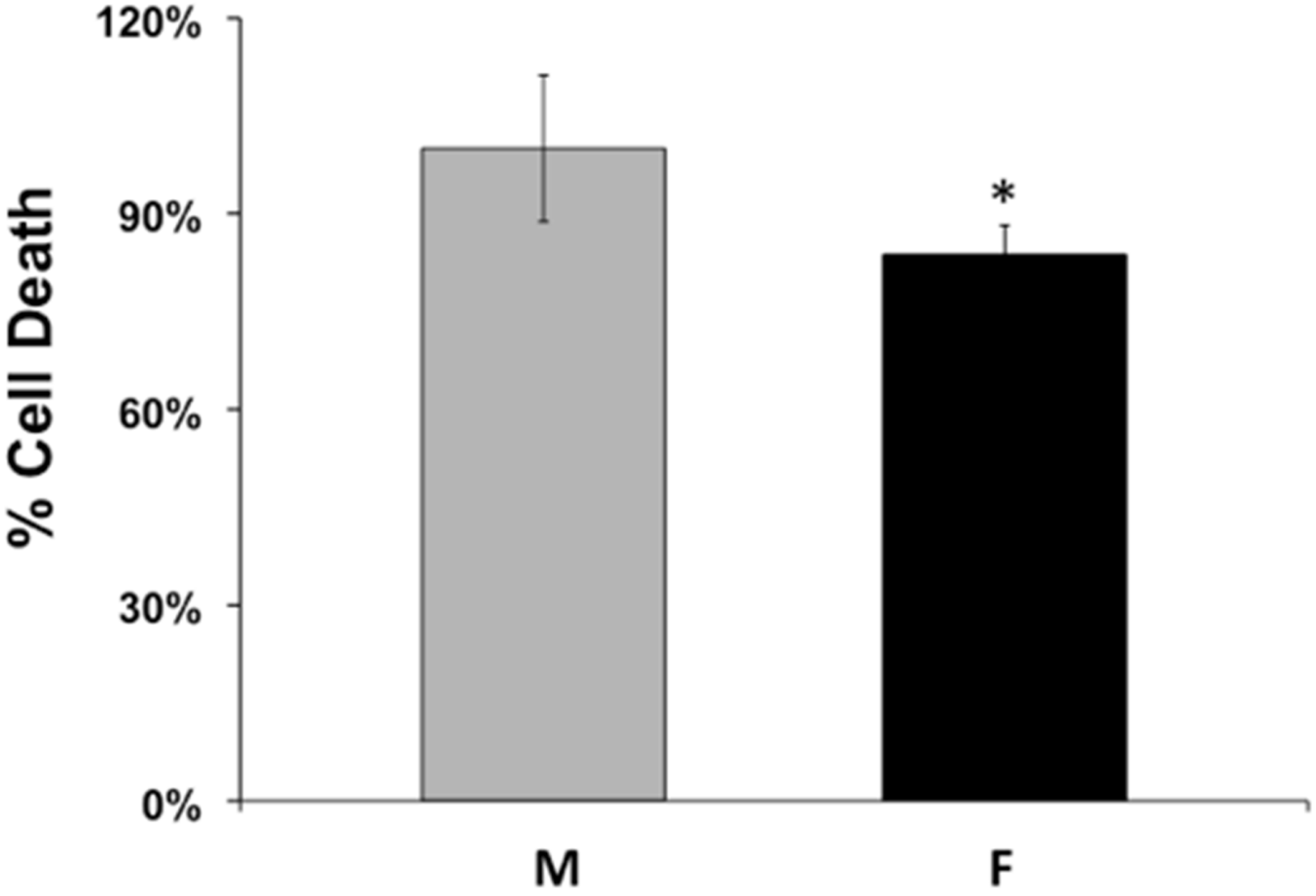

Figure 1. Cell death in primary cultured cortical neurons from male and female rat brains

Cortical neurons were cultured from embryonic day 18 male (M) and female (F) rat embryos and were subjected to OGD on day 10 in vitro. Cell death was measured by LDH release in neurons from male and female rats 24 hrs after reoxygenation. The graph depicts average cell death in neurons from male and female embryos expressed relative to the mean of cell death in males. (n=9, *p=0.0019).

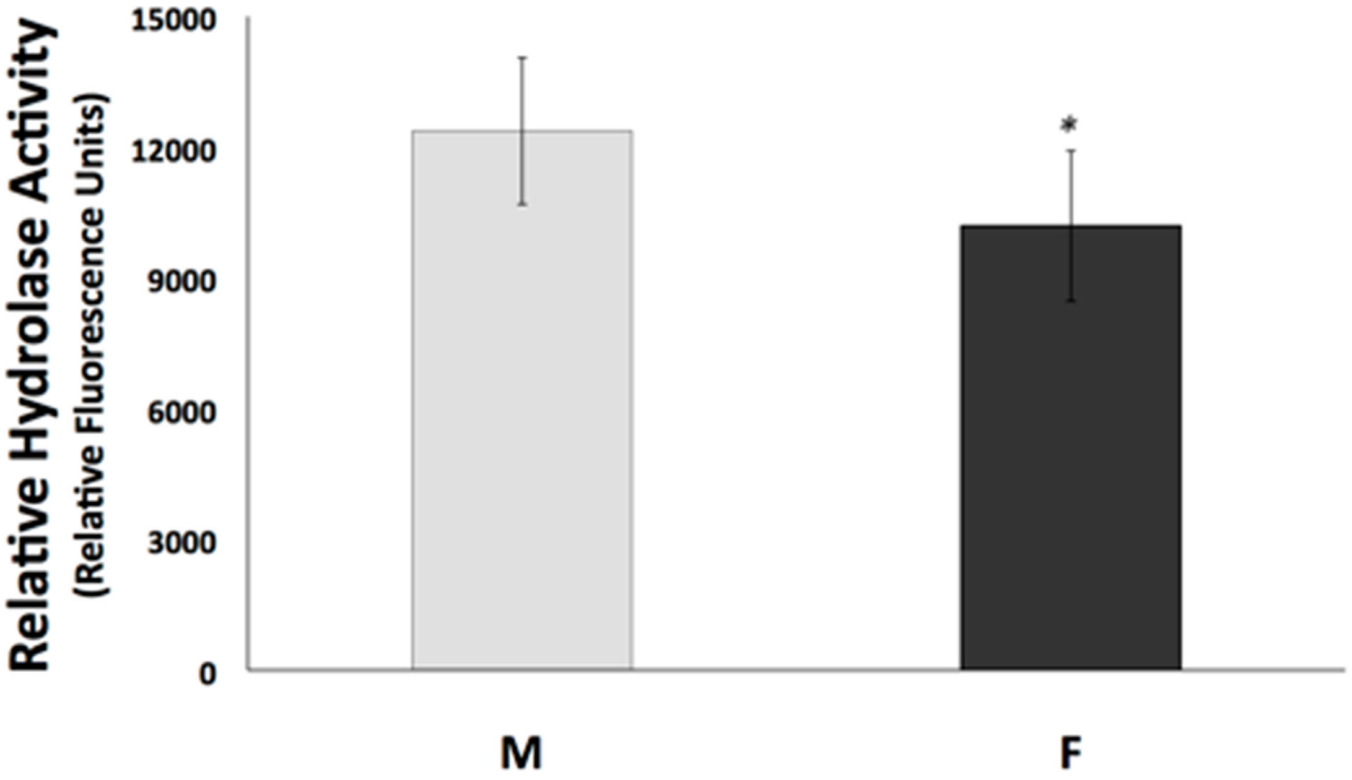

Figure 2. Hydrolase activity assay in cultured neurons from male and female rat brains

On day 10 in vitro, neuronal cultures from male (M) and female (F) rats were homogenized and incubated with epoxyfluor 7, which, when hydrolyzed by sEH, becomes a fluorescent compound. Fluorescence was measured using a fluorescent plate reader, and activity was normalized to reaction time and amount of protein. (n=6, *p=0.01)

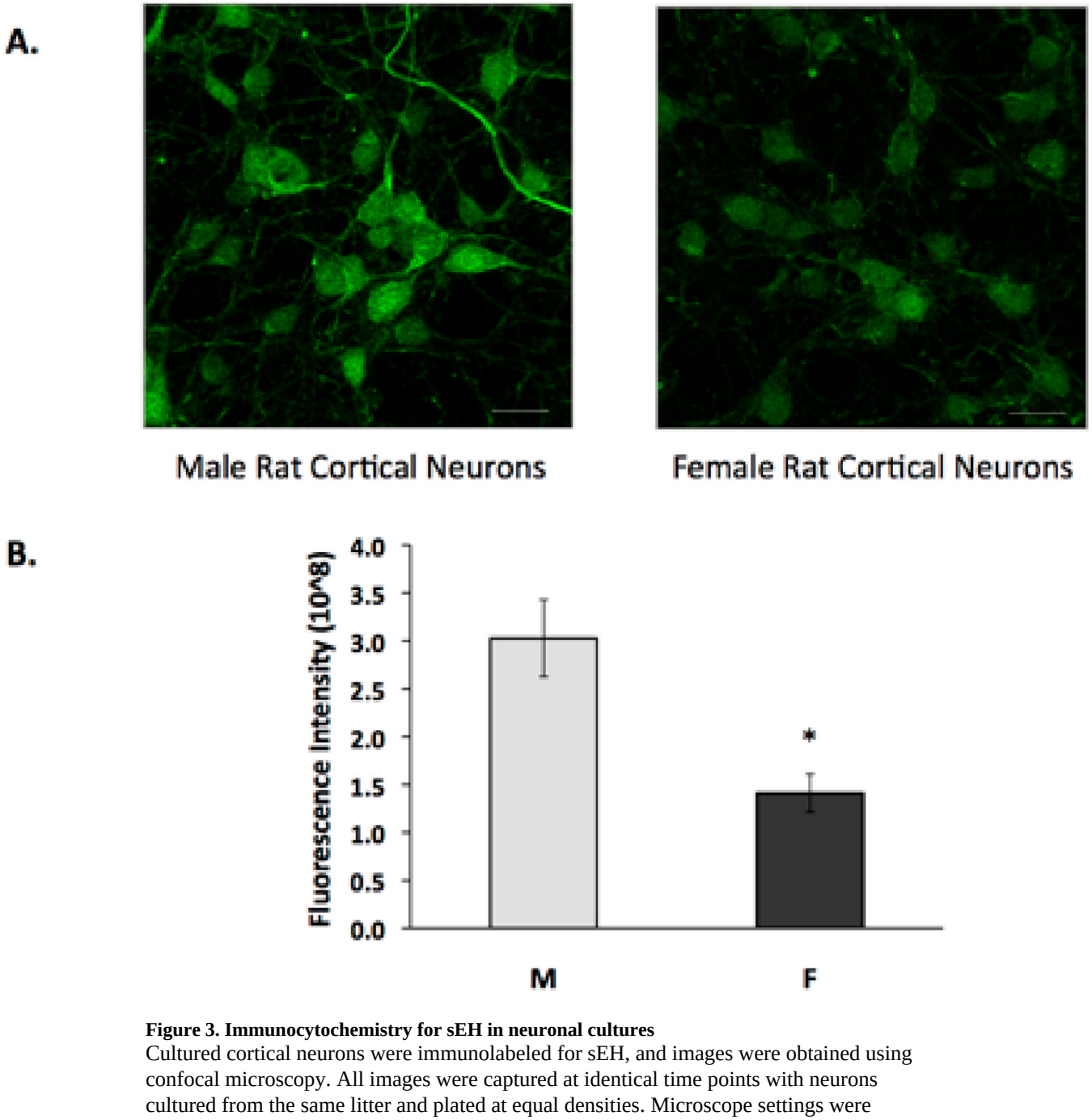

Figure 3. Immunocytochemistry for sEH in neuronal cultures

Cultured cortical neurons were immunolabeled for sEH, and images were obtained using confocal microscopy. All images were captured at identical time points with neurons cultured from the same litter and plated at equal densities. Microscope settings were identical for all imaged cells. **(A)** sEH is broadly expressed in cortical neurons as seen in representative confocal images. Scale bar, 20 μm. **(B)** The bar graph represents average total fluorescent intensity minus background intensity in neurons from males (M) and females (F). (n=4, *p=0.011) Images are representative of at least four biological replicates.

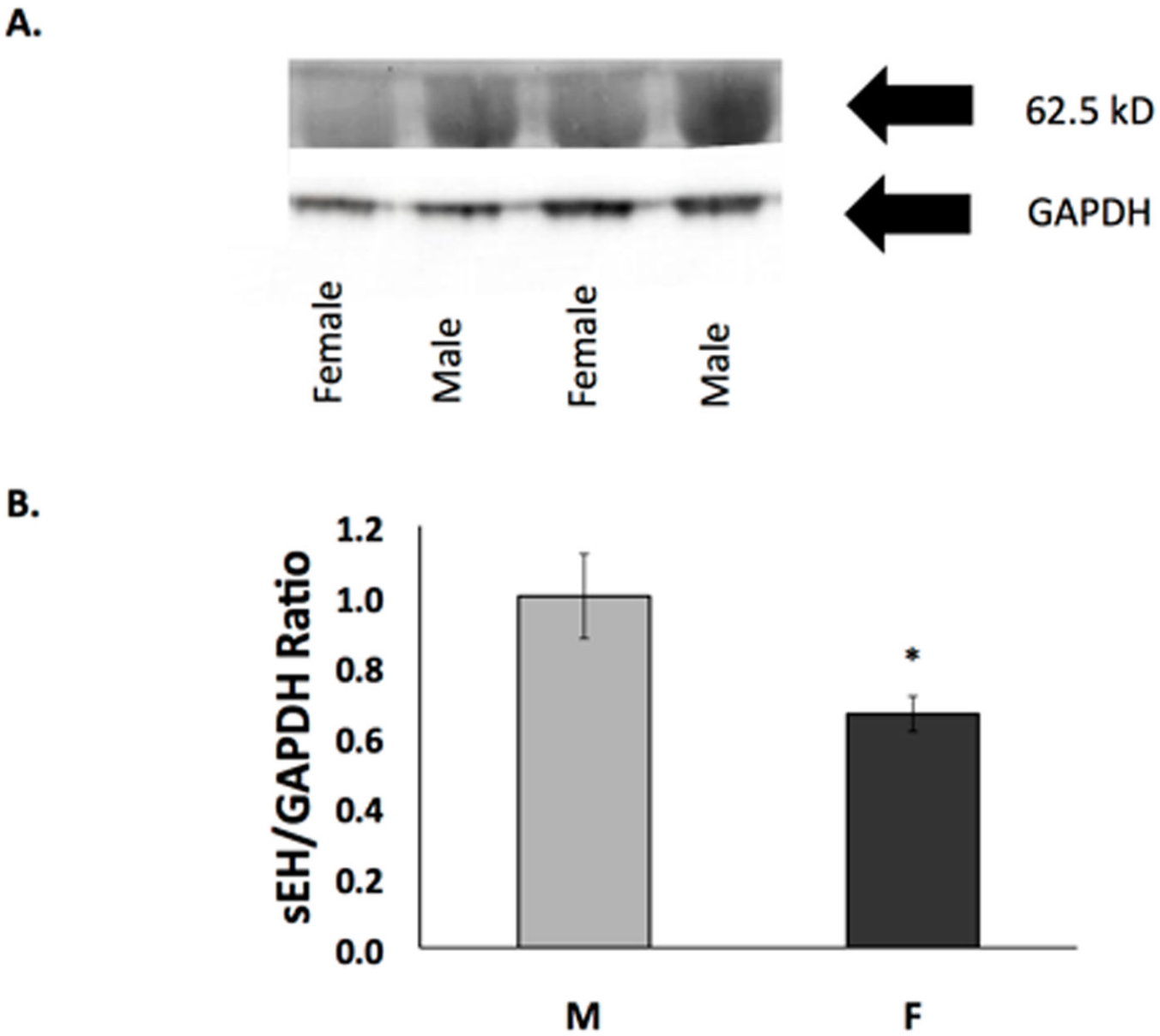

Figure 4. Western blot analysis of soluble epoxide hydrolase in cortical neurons from males and females

Western blot of protein extracts of neurons from males and females harvested after 10 days in vitro. sEH expression in cultured cortical neurons from males is significantly higher than in neurons from females. **(A)** Representative image of Western blot. Top panel is probed with anti-sEH antibody and bottom panel is probed with anti-GAPDH. **(B)** Quantification of Western blots of sEH normalized against GAPDH. Cells from males and females were paired within each experiment (n=4, *p=0.004)

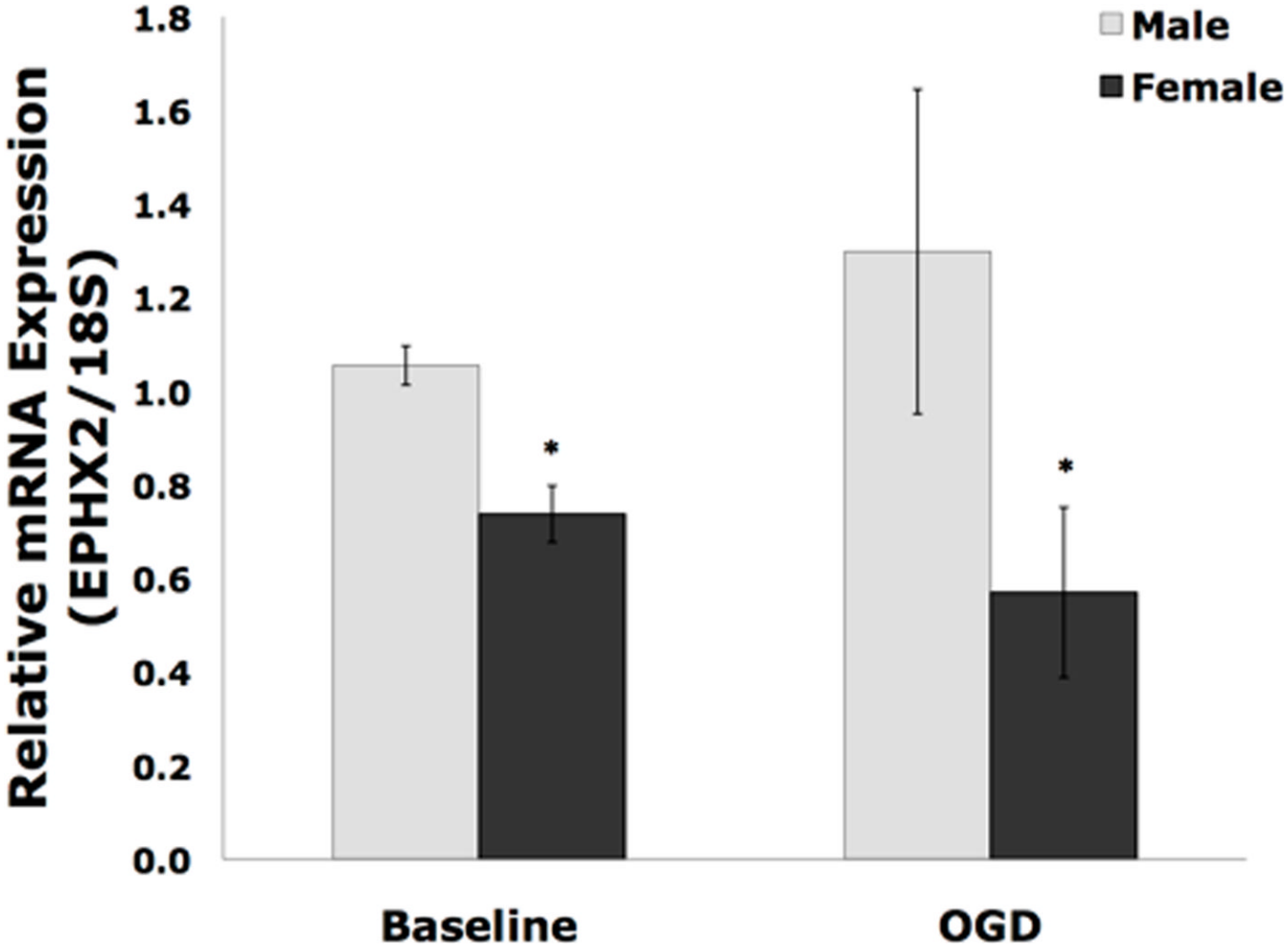

Figure 5. Real-time quantitative PCR for EPHX2 in cultured cortical neurons

Real-time quantitative PCR was performed on the 11th day in vitro on cortical cultured neurons from males (M) and females (F). Baseline represents untreated cells, while OGD represents cells that have undergone 2 hours OGD and then 24 hours reoxygenation. (n=6, *p<0.05) There were no differences between baseline and post-OGD values in either group.

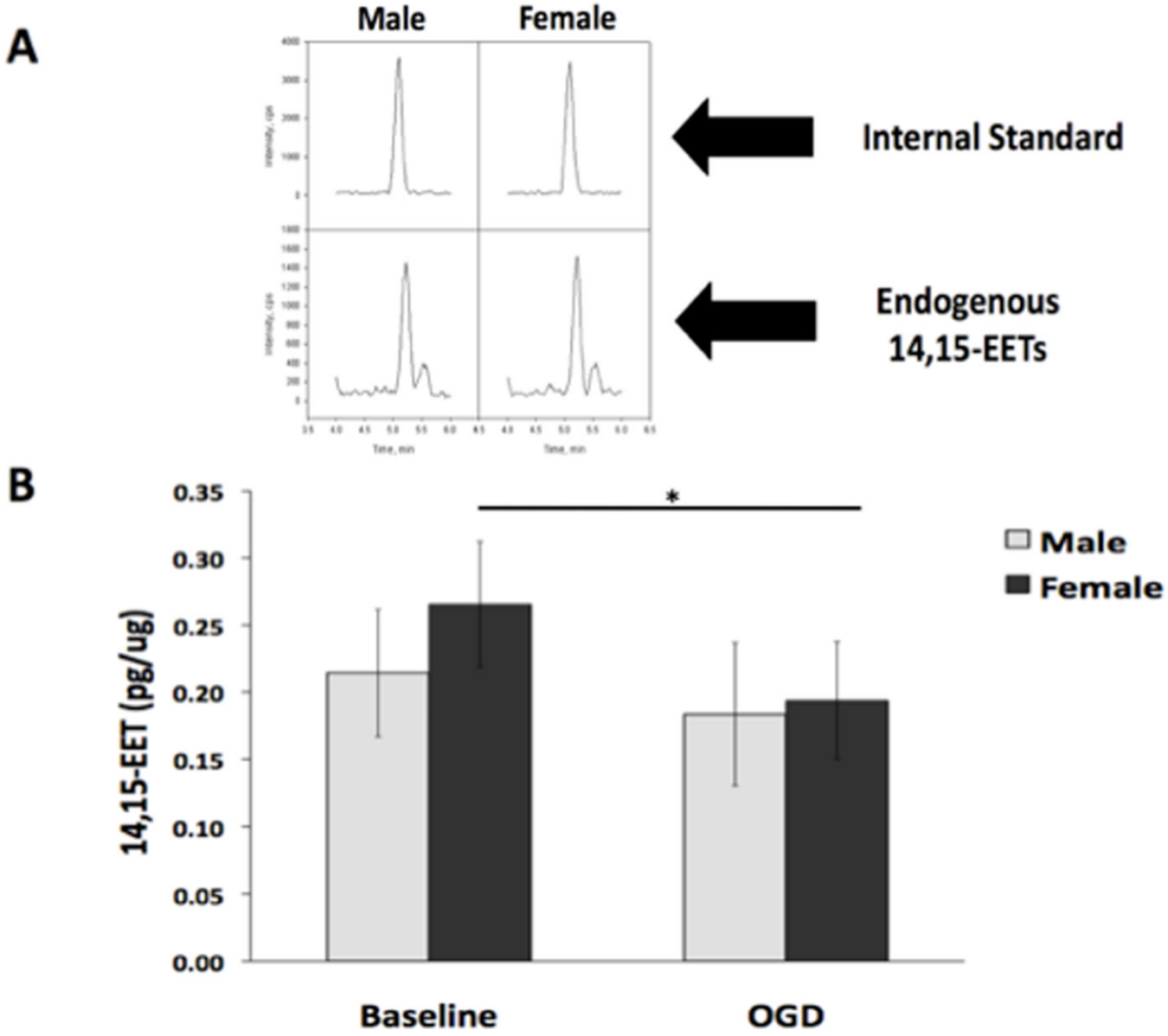

Figure 6. 14,15-EET was present in cells from males and females at baseline and after OGD

The concentration of EETs in neurons from males and females was evaluated using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in untreated (baseline) cells and cells subjected to 2 hours of OGD followed by 24 hours reoxygenation. **(A)** Representative 14,15-EET peaks obtained via LC-MS/MS in both male (M) and female (F) cells. The top panel represents the d8 14,15-EET internal standard. The bottom panel shows the endogenous 14, 15-EET peaks from the cells. The left two are from male cells and the right two are from female cells. The SRM transitions monitored were m/z 327.2 to 182.2 for the internal standard and the m/z for the 14,15 EET is 319.2 to 175. **(B)** The concentration of total 14,15-EET is not significantly different in cells from males and females at baseline (n=5, p=0.07). After OGD, 14,15-EET significantly decreased in cells from females, but not males. * Indicates a significant decrease in 14,15-EET after OGD in cells from females (n=5, *p=0.015).

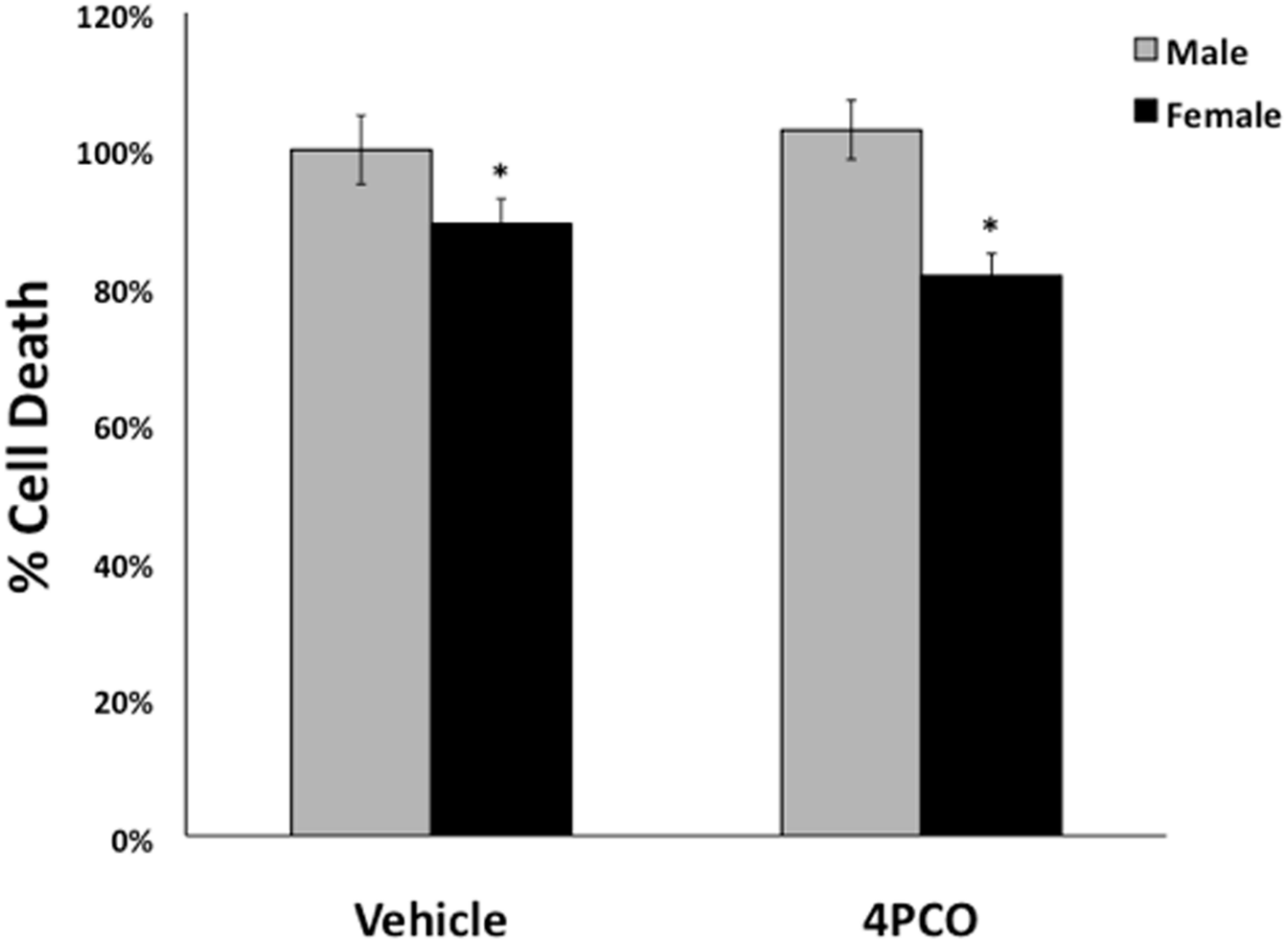

Figure 7. Inhibition of sEH using 4-PCO does not alter the difference in sensitivity to OGD between cells from males vs. females

On day 10 in vitro, cultured cortical neurons from males and females were treated with 4-Phenylchalcone oxide (4-PCO, 2 μM), an inhibitor of the hydrolase activity of soluble epoxide hydrolase, or vehicle (DMSO). Treatment was initiated 1 hour prior to OGD and continued throughout OGD and 24 hours of reoxygenation. Cells from females were more resistant to cell death in both vehicle and 4-PCO treated cells as expressed relative to the mean in cells from males (n=5, *p<0.05).

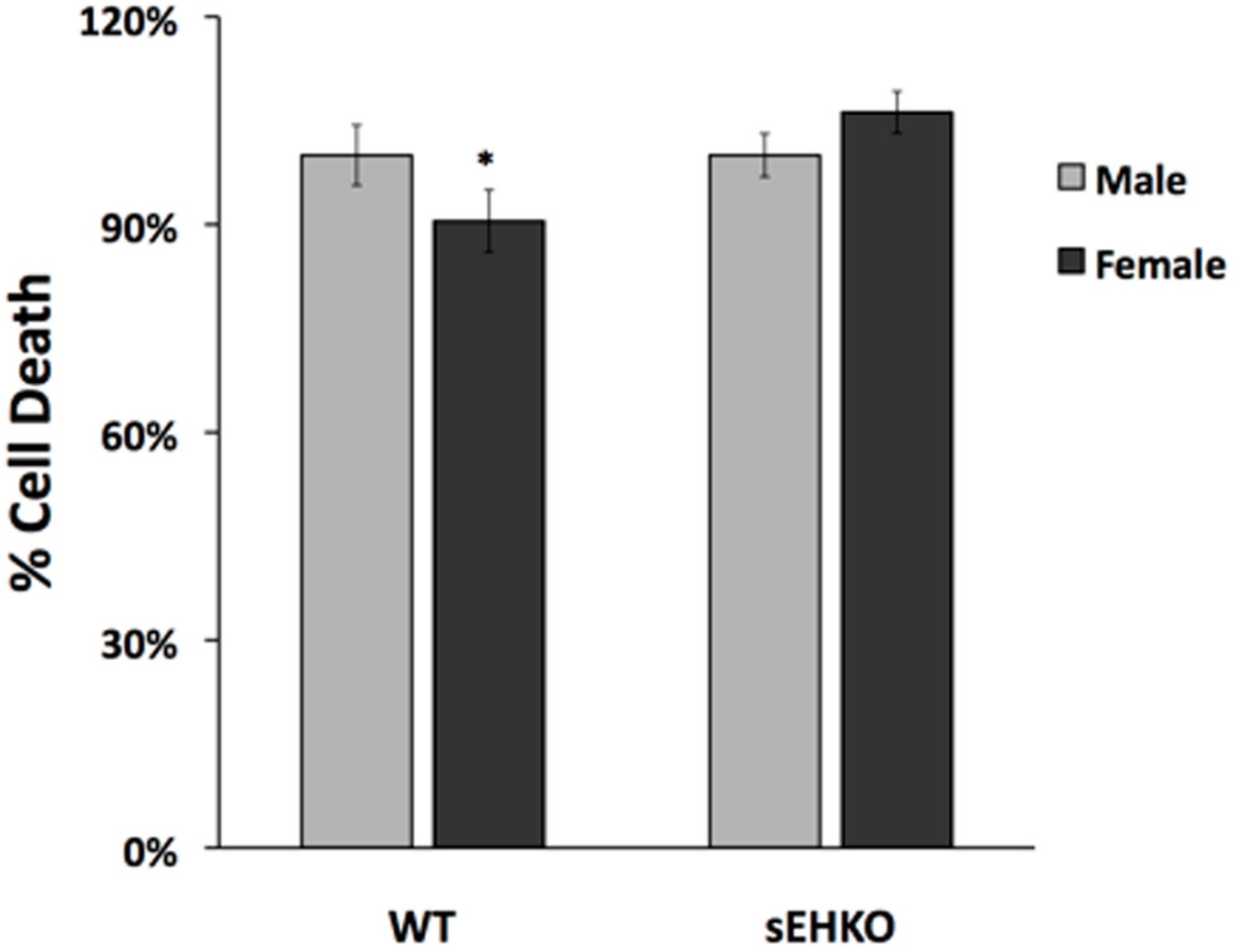

Figure 8. Knockout of sEH eliminates the difference in sensitivity to OGD between cells from males vs. females

Cortical neurons from wild-type (WT) and sEH knockout (sEHKO) mice were cultured and exposed to OGD on the 10th day in vitro. WT male mice had significantly more cell death after OGD compared with corresponding WT female mice, but this sex difference was not significant in the knockout mice. Average cell death in neurons from male and female embryos was expressed relative to the mean of cell death in males (n=6–8, *$p<0.05$).