Neuroscience 219 (2012) 183–191

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.**

Key words: acute stroke, EETs, brain ischemia, gender, soluble epoxide hydrolase.

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 has 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 (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 (sEH). 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 Alkayed, 2009), and sEH inhibition and gene deletion have also been shown to be protective against ischemic injury (Zhang et al., 2008, 2009). 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

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Abbreviations: DMEM, Dulbecco's-Modified Eagle Medium; DMSO, Dimethylsulfoxide; EETs, epoxyeicosatrienoic acids; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MCAO, middle cerebral artery occlusion; PLA2, phospholipase A2; OGD, oxygen-glucose deprivation; sEH, soluble epoxide hydrolase; sEHKO, sEH knockout.

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http://dx.doi.org/10.1016/j.neuroscience.2012.05.048

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.

EXPERIMENTAL PROCEDURE

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 the 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 (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 5-mL 0.5 mg/mL Papain (Worthington Biochemical Corporation, Lakewood, NJ) at 37 °C for 8 min. Papain was removed and tissue was washed twice with Trypsin Inhibitor (1 mg/mL, Trypsin Inhibitor, soybean, Sigma–Aldrich, St. Louis, MO) for 2 min. 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 1000 rpm for 5 min, 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.

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 seven generations, as previously described (Zhang et al., 2008). Homozygous sEHKO mice are viable, fertile, normal in size, and phenotypically identical to C57BL/6 mice. Mice were genotyped by polymerase chain reaction (PCR) as previously described (Sinal et al., 2000; Zhang et al., 2008).

Oxygen-glucose deprivation

To simulate ischemia, cells were subjected to OGD on DIV 10. OGD was performed as published in 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 ppm (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 h. After 2 h of OGD, DMEM was exchanged with prewarmed culture growth medium, and cells were returned to the normoxic incubator for 24 h. After 24 h 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. (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

sEH activity was determined using Epoxyfluor 7 (EP7), (Cayman Chemical Company Ann Arbor, Michigan, USA) as previously described (Jones et al., 2005). Cells were lysed in phosphate buffer saline (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 min in a black 96-well flat-bottomed 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% BSA (Sigma,