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Sex Differences in Cell Death

Hong Li, MD,[1] Scott Pin, BS,[1] Zhiyuan Zeng, BS,[2]
Michael M. Wang, MD, PhD,[3]
Katrin A. Andreasson, MD,[1]
and Louise D. McCullough, MD, PhD[2]

Female patients experience substantial neuroprotection after experimental stroke compared with male patients, a finding attributed to the protective effects of gonadal hormones. This study examined the response of male- and female-derived organotypic hippocampal slices to oxidative and excitotoxic injury. Both oxygen and glucose deprivation and *N*-methyl-D-aspartic acid exposure led to neuronal death; however, female-derived cultures sustained less injury than male-derived cultures. Cell death after oxygen and glucose deprivation was ameliorated in male cultures, but not female cultures, by the addition of 7-nitroindazole, a neuronal nitric oxide synthase inhibitor. These studies have relevance to researchers investigating neuroprotective agents in mixed sex experiments.

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Clinically, stroke is increasingly recognized as a sexually dimorphic disease. Most international databases demonstrate that women experience lower stroke incidence relative to men until advanced age.[1] This native neuroprotection is lost after menopause, and often is attributed to loss of estrogen.[2] It is equally well established that tissue damage and functional outcome after experimental brain injury are shaped by biological sex.[3–6] Emerging data suggest that cell death in brain may follow differing mechanistic paths depending on sex,[7,8] in addition to sex steroid exposure.

One major mechanism of ischemia-induced neuronal cell death results from overstimulation of neuronal nitric oxide synthase (nNOS) leading to enhanced production of nitric oxide (NO), consequent peroxynitrite formation, and nitrosative DNA damage. In response

From the [1]Departments of Neuropathology, Neuroscience, and Neurology, Johns Hopkins School of Medicine, Baltimore, MD; [2]Departments of Neurology and Neuroscience University of Connecticut Health Center, Farmington, CT; and the [3]Departments of Neurology and Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI.

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Address correspondence to Dr McCullough, Department of Neurology and Neuroscience, University of Connecticut Health Center, MC-1840, 263 Farmington Ave., Farmington CT, 06030-5332. E-mail: lmccullough@uchc.edu

to this DNA damage, the energy consuming DNA repair enzyme poly adenosine diphosphate ribose polymerase-1 (PARP-1) is activated. The extensive damage that occurs during ischemia leads to exuberant energy consumption, mobilization of mitochondrial proapoptotic molecules, and cell death.[9–13] However, the evidence establishing NO toxicity/PARP-1 activation as a major cytotoxic mechanism has accumulated from studies using exclusively male animals with deletions of nNOS ($nNOS^{-/-}$) or PARP ($PARP^{-/-}$) or mixed cell cultures.[9,12] Recently, we have demonstrated that significant sex differences exist in this basic cell death pathway after an ischemic insult in vivo. Genetic deletion of nNOS or PARP-1, although neuroprotective in male animals, led to an *exacerbation* of damage in female animals after middle cerebral artery occlusion.[14] Sex differences also exist in vitro. XY-derived (male) cortical and hippocampal neurons exhibited increased cell death after exposure to peroxynitrite or glutamate compared with those derived from XX (female) neurons.[15] This suggests that the male brain may be differentially susceptible to NO-mediated neurotoxicity. In this study, we tested the hypothesis that sex differences exist in hippocampal slice cultures, and that these are related to NO.

Materials and Methods

Preparation of Organotypic Cultures

Hippocampal organotypic slice cultures were prepared as described previously.[16] At 13 days in culture, medium was replaced with fresh medium containing propidium iodide (PI; 5μg/ml; Sigma, St. Louis, MO) for 24 hours, and basal PI fluorescence was imaged to assay spontaneous neuronal death ($t = 0$ hours). Induction of anoxia (see later) or exposure to *N*-methyl-D-aspartic acid (NMDA; 10mM; Sigma) was performed the following day in the presence or absence of either 30μM 3-bromo-7-nitroindazole (3-Br-7-NI; Sigma) or 10nm 17 β-estradiol (E2; for 7 days before oxygen and glucose deprivation [OGD][17,18]; Sigma), and PI fluorescence was calculated at 24 hours ($t = 24$ hours). Finally, the cultures were lethally exposed to 20*v*M NMDA overnight, and neuronal death was assayed by imaging and quantification of mean PI in the CA1 subregion of each hippocampal slice ($t = \max$).[15]

Oxygen and Glucose Deprivation

As described previously,[16] control and OGD-treated slices were included in each experiment with triplicate wells with 15 slices per condition for each experimental group (10–14 pups/group).

Nitric Oxide Production and Superoxide Dismutase Activity Measurements

Nitrite/nitrate and superoxide dismutase (SOD) activity (measured by generation of superoxide radicals by the xanthine/hypoxanthine reaction) were assayed at 2 and 24 hours after reoxygenation in additional OGD-exposed or control slices. NO(x) levels and SOD activity were determined by Nitrate/Nitrite Fluorometric Assay kit or SOD kit (Griess reagent; Cayman Chemicals, Ann Arbor, MI).

Sex Genotyping

Sex genotyping was accomplished by polymerase chain reaction analysis of genomic DNA from pup tails for the y-specific *Sry* gene (SRY3′ GAG AGA GGC ACA AGT TGG C/SRY5′ GCC TCC TGG AAA AAG GGC C) and *Myog* gene (MYOG ANTI TGG GCT GGG TGT TAG TCT TA/MYOG TTA CGT CCA TCG TGG ACA GC).

Statistical Analysis

Data are expressed as mean ± standard error of the mean as percentage of basal values from at least two to three separate

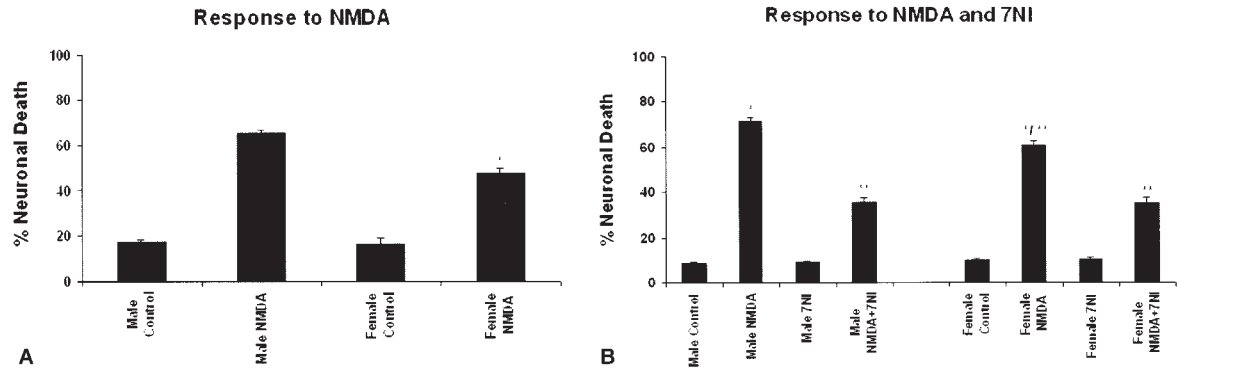

Fig 1. (A) Sex response to N*-methyl-D-aspartic acid (NMDA) toxicity. Baseline cell death was equivalent in male and female vehicle-treated cultures. NMDA exposure significantly increased neuronal cell death in both male and female cultures compared with control cultures; however, there was significant neuroprotection evident in female-derived cultures (*$p < 0.01$*) after NMDA exposure compared with male cultures. (B) Response of sex-specific cultures to NMDA toxicity after treatment with the nitric oxide inhibitor 7-nitroindazole (7-NI). No differences were seen in control or 7-NI vehicle-treated cultures. After NMDA, male slices showed enhanced toxicity compared with both male control (*$p < 0.05$*) and female NMDA-treated culture slices (*$p < 0.05$*; **$p < 0.05$*). After preexposure to 7-NI, both male and female slices demonstrated robust neuroprotection (**$p < 0.05$*).*

experiments. Data were analyzed by one-way analysis of variance followed by Newman–Keuls post hoc test. Values were considered statistically significant at $^{*}p < 0.05$.

Results

No Differences Exist in Basal Cell Death Rates

Baseline neuronal cell death (measured as baseline PI florescence) did not differ in XX and XY control cultures (Figs 1A and 2A) or in sex-selected control cultures treated with 7-nitroindazole (7-NI) or E2 (see Figs 1B, 2B, and 2C). There were no gross morphological differences between XY and XX hippocampal slices.

Male Slices Had Significantly Enhanced Cell Death after Insults

Cell death markedly increased after NMDA ($p < 0.001$) exposure in both male and female cultures compared with sex-matched, vehicle-treated, control cultures (see Fig 1A). A significant sex effect was seen after NMDA ($p < 0.05$; see Fig 1A) and OGD ($p < 0.001$; see Fig 2A) exposure wherein XY-derived cells exhibited significant increases in neuronal cell death compared with female cells. This difference was especially prominent after OGD (see Fig 2A).

Neuroprotective Effects of 7-Nitroindazole Were Seen after N*-methyl-*D*-aspartic Acid Toxicity*

Immediate preexposure to 7-NI reduced the neuronal damage induced by NMDA toxicity in *both* male- and female-derived cultures (see Fig 1B). After treatment with 7-NI, female cultures had equivalent amounts of cell death compared with their male counterparts (male: 35.4 ± 2.4%; female: 35.3 ± 2.2%), although the absolute reduction in cell injury was less in female cultures because they were initially "protected" (male: 71 ± 1.9%; female: 60.8 ± 2%). Interestingly, although female slices showed a significant reduction in neuronal death after nNOS inhibition in our NMDA model (see Fig 1B), no protective effect of NOS inhibition was seen in female slices (vehicle: 53 ± 3%; 7-NI: 58 ± 3.3%) after OGD exposure (see Fig 2B).

Neuroprotective Effects of 17 β-Estradiol Were Seen in Both Male and Female Cultures

Estrogen pretreatment reduced damage in both XX and XY cultures (see Fig 2C) after OGD. This effect was more prominent in male-derived slices. Male and female E2-treated slices demonstrated equivalent neuronal cell death (male: 39.9 ± 2%; female: 40.3 ± 2%) after OGD.

Male and Female Slices Had Equivalent Reductions in Superoxide Dismutase Activity

SOD activity decreased after OGD in both sexes (because of its consumption of superoxide radical gener-

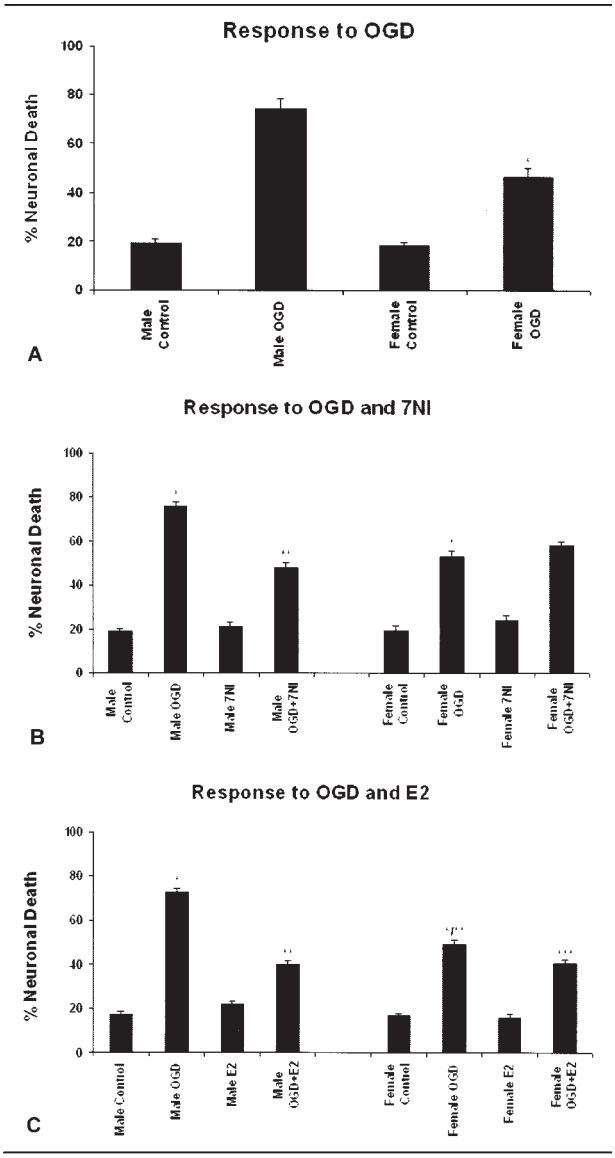

Fig 2. (A) Sex response to oxygen and glucose deprivation (OGD) injury. Baseline cell death was equivalent in male and female control cultures. OGD exposure significantly increased neuronal cell death in both male and female cultures compared with control cultures. There was significant intrinsic neuroprotection in female-derived cultures ($^{**}p < 0.01$) after OGD exposure compared with male cultures. (B) Sex response to 7-nitroindazole (7-NI) after OGD-induced neuronal injury. Pretreatment with 7-NI significantly reduced neuronal cell death compared with vehicle-treated cultures ($^{**}p < 0.01$) in male cultures, yet had no effect in female cultures. Female animals again demonstrated a significant intrinsic neuroprotective effect compared with male animals ($^{*}p < 0.01$). This was no longer evident after treatment with 7-NI. (C) Sex response after treatment with estrogen. 17-β estradiol (E2) was administered to cultures 7 days before OGD. Male cells demonstrated enhanced cell death after OGD ($^{*}p < 0.01$) compared with female cells. However, both male and female cells were protected by E2 ($^{**}p < 0.05$), although the effect was more robust in male cells ($^{***}p < 0.05$).

ated by OGD). Total SOD activity decreased from 4.36 ± 0.31μg/ml to 2.79 ± 0.20μg/ml in female animals and from 4.92 ± 0.72μg/ml to 2.38 ± 0.31μg/ml in male animals 2 hours after OGD. Similar decrements were seen at 24 hours.

Markers of Nitrosative Stress Were Increased in Male Slices

The concentrations of nitrite + nitrate, nitrite, and nitrate were increased after OGD in both groups, but they were significantly greater in male animals at both 2 and 24 hours after OGD ($p < 0.05$; Fig 3).

Discussion

This article demonstrates three important findings. First, there is a consistent sex difference after either OGD or NMDA exposure in hippocampal slice cultures demonstrating an intrinsic neuroprotection in female-derived cells. Second, acute treatment with a nNOS inhibitor ameliorates cell death in both male and female neurons after NMDA exposure, yet it has no effect in females after OGD. Third, although there are no sex-based differences in SOD activity, by-products of NO production are increased in male-derived cells after OGD.

Recent studies have shown that female-derived neuronal cultures have a distinct survival advantage over those derived from male cultures.[8,15] XY neurons are more susceptible to nitrosative stress (via peroxynitrite) and excitotoxic cell death (via glutamate and NMDA toxicity) than female cells. Male cells demonstrated prominent increases in the nuclear translocation of apoptosis-inducing factor,[15] a caspase-independent cell death pathway,[10] after these insults. In contrast, female cells were differentially susceptible to staurosporine-induced apoptotic cell death, a process mediated by caspase activation.[15] In vivo, female mice are insensitive to the pharmacological loss or genetic deletion of nNOS, strategies that are dramatically neuroprotective in male mice.[14] This suggests that molecular cell death pathways are sexually dimorphic in brain.

In this article, we have demonstrated similar sex disparities in hippocampal slices at a time when hormonal differences between the sexes are minimal.[19] Intrinsic female neuroprotection is most strikingly demonstrated after OGD. This model may mimic in vivo ischemia more closely than NMDA as slices are exposed to both oxidative *and* nutrient deprivation. NMDA toxicity may activate selective cell death pathways that are less damaging to the female brain. Alternatively, OGD ex-

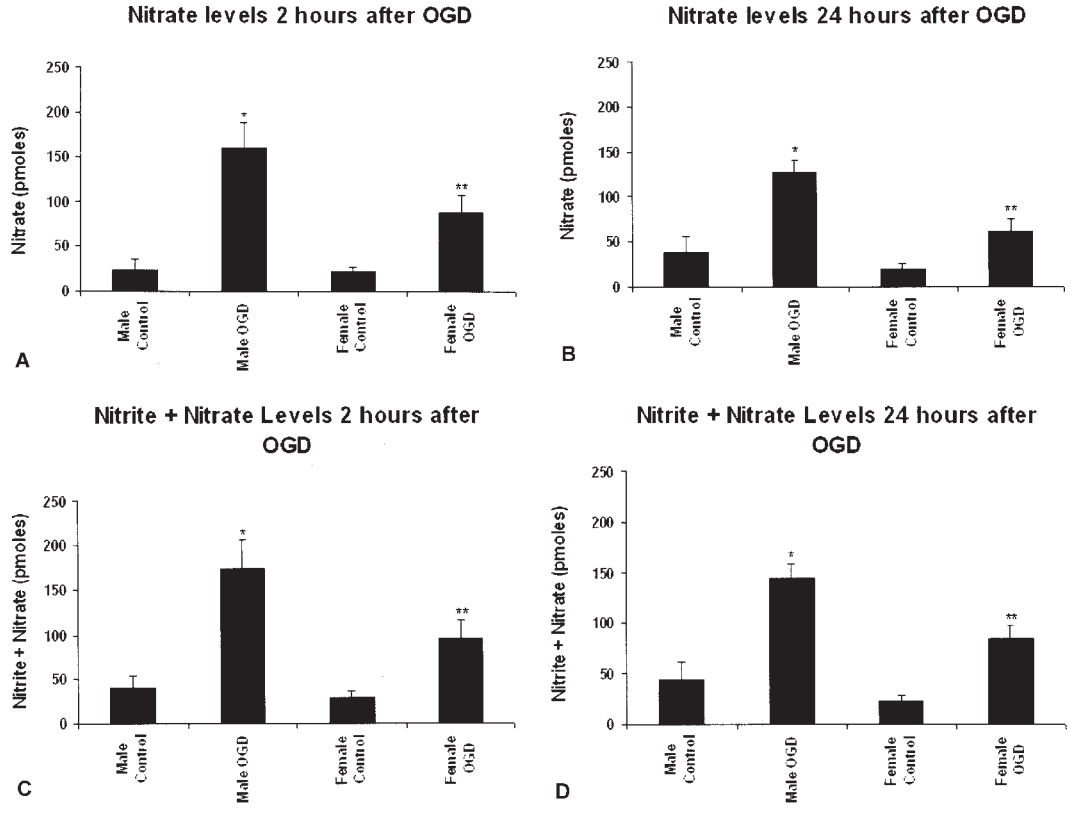

Fig 3. Production of nitric oxide by-products in male- and female-derived hippocampal slices. Significant increases in nitrate levels were seen after both 2 (A) and 24 hours (B) of oxygen and glucose deprivation (OGD). Combined nitrate and nitrite levels also were significantly greater in male cultures at both 2 (C) and 24 hours (D) after OGD injury.

posure may trigger other downstream effectors in addition to that of NO. The finding that XX slices are unresponsive to nNOS inhibition after OGD, yet remain responsive to nNOS inhibition after NMDA challenge, may reflect differences in the type and severity of injury in the two models. Differences in the level of NO production by each of these insults (with OGD producing less NO in female animals than NMDA injury) are also possible. We have demonstrated that markers of NO production are greater in male-derived slices exposed to OGD than in female slices, which may explain the greater effect of NO inhibition in male slices. Our recent in vivo findings also confirm that female animals do not benefit from manipulations that reduce NO.[14] Because these studies were aimed at determining if sex differences exist in a culture model designed to mimic stroke, our findings in the OGD model may be relevant. Clearly, the cell death pathways for each of these models (NMDA and OGD) will need to be explored further.

As has been shown by others, estrogen is neuroprotective in the slice model.[17] The neuroprotective benefit is much more modest in female slices, an effect likely related to the lower baseline cell death after OGD in XX slices (see Fig 2C). Recent work has shown similar results after hypoxia in male neuronal cultures. Estrogen treatment preferentially protects male cells, possibly related to changes in membrane estrogen receptor ratios.[20]

These results have implications for preclinical experimental studies and clinicians treating stroke patients. Sex effects are being recognized clinically as well, as tissue plasminogen activator (the one approved therapy for stroke) is more efficacious at clot lysis in female stroke patients.[21] Targeting sex-selective cell death may enhance our ability to salvage ischemic brain.

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