Sex Differences in Caspase Activation After Stroke

Fudong Liu, MD; Zhong Li, MD, PhD; Jun Li, PhD; Chad Siegel, PhD; Rongwen Yuan, MD, PhD; Louise D. McCullough, MD, PhD

Background and Purpose—Over the past 5 years, experimental data have emerged that ischemia-induced cell death pathways may differ in males and females. Cell death in males is triggered by poly(ADP-ribose) polymerase activation and nuclear translocation of apoptosis-inducing factor. We have previously shown that interference with this pathway benefits males but not females after an experimental stroke. In contrast, caspase activation may be the major pathway activated after ischemic injury in females. The aim of this study is to examine whether sex differences exist in caspase activation in adult mice after stroke and to determine if interference with stroke-induced caspase activation preferentially protects females.

Methods—Focal stroke was induced by reversible middle cerebral artery occlusion (90 minutes) in young and aging C57BL/6 mice of both sexes. The pan-caspase inhibitor, quinoline-Val-Asp(Ome)-CH2-O-phenoxy was administered at reperfusion. Histological outcomes were assessed 48 hours after middle cerebral artery occlusion. Separate cohorts were used for protein analysis of key cell death proteins, including caspase-3, caspase-8, cytochrome C, and apoptosis-inducing factor.

Results—Drug-treated female mice had significantly decreased infarct volumes and improved neurological deficits after stroke compared to vehicle-treated mice. Quinoline-Val-Asp(Ome)-CH2-O-phenoxy administration had no effect in male mice. The expression of cytochrome C and nuclear caspase-8 levels were increased in females after stroke.

Conclusions—Female mice had an early release of cytochrome C and enhanced caspase activation after middle cerebral artery occlusion. Caspase inhibition benefited females but not males. Sex differences exist in both the response to ischemic injury and the efficacy of neuroprotective agents. (*Stroke.* 2009;40:1842-1848.)

Key Words: caspase □ cytochrome C □ middle cerebral artery occlusion □ sex differences □ stroke

We have known for some time that the epidemiology of human stroke is sexually dimorphic until late in life, well beyond the years of reproductive senescence and menopause. The principal mammalian estrogen (17-β estradiol or E2) is neuroprotective in many types of brain injury and has been the major focus of investigation over the past several decades.1–4 However, despite preclinical and observational evidence of a protective role for estrogen, recent randomized clinical trials such as the Women’s Health Initiative have failed to translate the beneficial effects of estrogen into a viable therapy for stroke prevention in postmenopausal women.5 In addition, women continue to have a decreased incidence of stroke compared to men well beyond (≥20 years) menopause, suggesting that not all the observed “female protection” is mediated by gonadal steroids.6,7 Ischemic sexual dimorphism also exists in the outcome from brain injury in subjects before puberty, when estrogen is less likely to be involved.8 Over the past 5 years, data are emerging that suggest that the cell death pathways activated after experimental stroke are influenced by biological sex as well as hormone exposure. One well-investigated cell death pathway is mediated by neuronal nitric oxide (NO). Increasing levels of NO and peroxynitrite lead to DNA damage and activation of the DNA repair enzyme poly(ADPribose) polymerase-1 (PARP-1). Over activation of PARP triggers the translocation of apoptosis-inducing factor (AIF) from the mitochondria to the nucleus9–14 and leads to caspase-independent apoptosis.11 Surprisingly, this basic ischemic death pathway shows dramatic sexual dimorphism. Males benefit from inhibition or deletion of neuronal NO synthase or PARP-1, whereas females have a striking exacerbation of injury.15

Sex specificity can also be modeled in cell culture when background sex steroids are removed from the media. After cytotoxic challenge, programmed cell death in cortical neurons proceeded predominately via an AIF-dependent pathway in male (XY) neurons vs a cytochrome C-dependent pathway in female (XX) neurons.16 Large-scale DNA fragments (50-kbp, the biochemical hallmark of AIF-mediated cell death17) were increased in XY neurons after ONOO− exposure. In
contrast, mitochondrial release of cytochrome C, caspase-3 proteolysis, and oligonucleosomal DNA fragmentation (the phenotypic hallmarks of caspase-mediated programmed cell death) were more prominent in female neurons.

Recent work in neonatal animals suggests that females are exquisitely sensitive to caspase-mediated cell death, because caspase inhibition dramatically reduced injury after a hypoxic-ischemic insult. Acknowledging that rates of apoptosis differ among developmental ages, it is not known whether similar sex differences in caspase-mediated cell death exist in adult animals. Quinoline-Val-Asp(OMe)-CH2-O-phenoxyl (Q-Vn-OPh) is a novel, cell-permeable, broad-spectrum caspase inhibitor that has a potent effect on suppressing caspase-induced apoptosis. In this study we administered Q-Vn-OPh to test the hypothesis that caspase-dependent cell death pathways are preferentially activated in the female brain after experimental stroke.

Materials and Methods

Animals

C57BL/6 mice were purchased from Charles River Laboratories (Willimantic, Conn, USA). All experiments were performed according to NIH guidelines for the care and use of animals in research and under protocols approved by the University of Connecticut Health Center Animal Care and Use Committee. Both young mice (9–12 weeks; 21–25 grams) and aging mice (16 months; males 35–50 grams, females 26–38 grams) of both sexes were used.

Ovariectomy and E2 Treatment

In ovariectomized (Ovx) females, the ovaries were surgically removed 10 days before middle cerebral artery occlusion (MCAO) as described previously. In E2-treated mice, 17β-estradiol was delivered by subcutaneous SILASTIC capsule (0.062-inch inner diameter; 0.125-inch outer diameter) filled with 0.035 mL of 17β-estradiol (180 µg/mL; Sigma) in sesame oil implanted at the time of ovariectomy. Control mice were implanted with oil-containing capsules. Serum 17β-estradiol was measured by enzyme-linked immunosorbent assay (IBL, Hamburg).

Focal Cerebral Ischemic Model

Focal transient cerebral ischemia was induced by MCAO (0.21-mm suture) for 90 minutes, followed by reperfusion as described previously. In E2-treated mice, 17β-estradiol was delivered by subcutaneous SILASTIC capsule (0.062-inch inner diameter; 0.125-inch outer diameter) filled with 0.035 mL of 17β-estradiol (180 µg/mL; Sigma) in sesame oil implanted at the time of ovariectomy. Control mice were implanted with oil-containing capsules. Serum 17β-estradiol was measured by enzyme-linked immunosorbent assay (IBL, Hamburg).

Behavioral Scoring

Neurological deficits were scored at 1.5 hours, 6 hours, or 48 hours after stroke. The scoring system was as follows: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling.

Drug Administration

Q-Vn-OPh (MP Biomedicals) was dissolved in DMSO and further diluted with sterile phosphate-buffered saline. Q-Vn-OPh (5 to 20 mg/kg) was injected intraperitoneally at reperfusion (90 minutes after occlusion). Control mice were injected with vehicle alone.

Histological Assessment

At 48 hours after stroke, the mice were euthanized and the brains were removed and cut into 5 2-mm slices. The slices were stained with 1.5% 2,3,5-triphenyltetrazolium solution at 37°C for 10 minutes. The stained slices were fixed with 4% formalin, images were digitallyized, and the infarct volumes (corrected for edema) were analyzed using Sigmascan Pro5 as previously described.

Subcellular Fractionation

Brain samples were obtained by rapid removal of the brain, with removal of the cerebellum/occipital pole and olfactory/frontal pole. Samples were homogenized in Dounce homogenizers with cold lysis solution (10 mmol/L Tris-HCl, pH 7.5; 5 mmol/L MgCl2; 0.1 mmol/L EDTA; 1.5 mmol/L CaCl2; 0.25 mmol/L sucrose; 1 mol/L DTT; 10% Triton X-100; 1:50 protease inhibitor). Homogenates were centrifuged at 800g for 10 minutes at 4°C. The pellet (P1) contained the nuclear fraction; the supernatant was further centrifuged at 14 000g for 30 minutes (cytosolic and mitochondrial fractions). P1 was resuspended in lysis buffer and run through a sucrose gradient composed of 1.8 mol/L and 2.3 mol/L sucrose with ultracentrifugation at 30 000g for 45 minutes. The pellets (P2) were collected with nuclei pure storage buffer (Sigma-Aldrich) and centrifuged at 2300 rpm for 10 minutes. The pellets were resolved with extraction buffer (Sigma-Aldrich), sonicated for 10 seconds 3 times, and stored at −80°C. All samples represent pooled samples (5 strokes; 2 sham/group). Because of the nuclear extraction process, the amount of nuclear protein obtained from a single mouse was so small that pooling samples from each group was required.

Western Blots

The fractionated protein concentration was determined by BCA Protein Assay Kit (Thermo Fisher Scientific Inc) and subjected to Western blotting as previously described. Sample proteins were resolved on 4% to 20% SDS electrophoresis gels and transferred to a polyvinylidene difluoride membrane. Cytochrome C, AIF, caspase-3, caspase-8, and caspase-9 were detected using corresponding antibodies from cell signaling (1:1000). Beta-actin (1:1000; Sigma), β-tubulin (1:1000; Santa Cruz), and histone H3 (1:4000; Sigma, for nuclear fraction) were used as loading controls. All blots were incubated overnight in primary antibodies at 4°C in Tris-buffered saline containing 4% bovine serum albumin and 0.1% Tween 20. Secondary antibodies (goat antirabbit IgG 1:5000, goat antimouse IgG 1:2000, donkey antigoat IgG 1:1000; Santa Cruz) were diluted, and ECL detection kit (Amersham Biosciences) was used for signal detection. The densitometry of Western blotting images was performed with computer software (Scion Image). Each sample was performed in triplicate.

Statistics

Data from individual experiments were presented as mean±SEM. One-way analysis of variance (with Turkey post hoc correction, when appropriate) was used for the comparison of the means between the experimental groups. P<0.05 was considered statistically significant. Induction of ischemia and behavioral and histological assessments were performed by an investigator blinded to drug treatment.

Results

Sex Differences in Caspase Activation

To determine if differences in caspase activation were present between male and female mice after MCAO, we began by assessing levels of caspase-8 to evaluate the extrinsic pathway, and caspase-9 to assess the apoptosome/intrinsic pathway. No differences were seen in caspase-9 or its cleavage products in male and intact female mice (data not shown) at any time point in the nuclear, cytoplasmic, or mitochondrial fractions. However, nuclear caspase-8 levels varied dramatically by gender (Figure 1A,B). Cleaved caspase-8 (full-length 57 kDa; cleaved forms 43, 18kDa) steadily increased in the nuclear fraction of females after stroke. E2 levels were not significantly different between drug- and vehicle-treated mice.
To ensure that the confounding effect of estrogen was removed, we subsequently examined intact, Ovx, and E2-replaced mice in our Q-VD-OPh studies. Serum 17β-estradiol levels were equivalent within the drug- and vehicle-treated young male, Ovx plus oil-treated female, intact female, and Ovx plus E2-treated female groups. Levels in the Ovx plus E2-treated female mice were significantly higher than those in any other group. As expected, the estrogen levels in intact female were significantly higher than those in either the vehicle-treated young male or Ovx plus oil-treated female group (Figure 2A). E2 levels were not significantly different in male and female aging mice (Figure 2B), confirming loss of estrogen in aging females.

**Stroke Outcomes in Young and Aging Mice**

We first administered 5 mg/kg of Q-VD-OPh intraperitoneally to young male, Ovx plus oil-treated female, intact female, and Ovx plus E2-treated female mice. Drug-treated female mice had significant reductions in infarct compared to vehicle-treated mice regardless of their hormone status: Ovx plus oil-treated female (total: vehicle 53.9±2.1%, n=6 vs drug 31.0±3.3%, n=6; P<0.05), intact female (total: vehicle 43.8±3.4%, n=9 vs drug 24.4±6.4%, n=6; P<0.05), and Ovx plus E2-treated female (total: vehicle 35.0±3.1%, n=7 vs drug 20.0±5.4%, n=7; P<0.05) groups (Figure 2C). As expected, the E2-replaced and intact female mice had smaller strokes than males or Ovx females (P<0.05), consistent with estrogen’s known neuroprotective effects. No neuroprotective effect of Q-VD-OPh was seen in male animals (total: vehicle 53.9±2.2%, n=6 vs drug 54.6±2.0%, n=6; P>0.05).

To determine whether administration of a higher dose of Q-VD-OPh could protect males and lead to a more robust decrease of infarct volumes in females, we injected a high
dose (20 mg/kg intraperitoneally) of Q-VD-OPh to male and Ovx female mice. Q-VD-OPh–treated Ovx females had significantly smaller infarct volumes than vehicle-treated Ovx females or males. There were no differences in infarct volumes between the low-dose and the high-dose Q-VD-OPh administration in males or females (Figure 2C).

Because caspase-induced cell death may be developmentally regulated, we also administered Q-VD-OPh to aging mice to determine if the sex-dependent effect of Q-VD-OPh exists in aging animals. Male and female aging mice were treated with 10 mg/kg Q-VD-OPh and stroke outcome was assessed at 48 hours. There was no significant difference in infarct volume between vehicle- and drug-treated male aging mice; however, Q-VD-OPh continued to have a significant neuroprotective effect in females (total: vehicle 48.5% vs drug 28.4%; n = 6 vs drug 28.4 ± 5.7%; n = 6; P < 0.05; Figure 2D). Aging male mice had smaller infarcts than young male mice, yet they had higher mortality and neurological deficits (Table 1). Mortality within 48 hours was 6% (6.3% in vehicle- and 5.6% in drug-treated group) for young and 27% for aging mice (30% in vehicle- and 25% in drug-treated group).

No differences in pH, pO2, pCO2, blood glucose, mean arterial pressure, or laser Doppler flowmetry were seen between vehicle- and drug-treated mice of either gender and either age (aging mice not shown; Table 2). Neurological deficits were significantly improved at 6 hours after stroke in drug-treated female young and aging mice, but not in vehicle-treated mice of either gender or either age (Table 2).

Cytosolic Cytochrome C Increases Early in Female Mice After Stroke
We then examined the expression of cytochrome C in the cytosol and AIF translocation to the nucleus after stroke. The expression of cytochrome C dramatically increased in vehicle-treated female mice compared to males at 6 hours after stroke, and Q-VD-OPh treatment attenuated this increase. Males had increased levels of cytochrome C 12 hours after stroke compared to females, and the levels were even higher with Q-VD-OPh treatment (Figure 3A,B). Both male and female mice had increased nuclear translocation of AIF after stroke; treatment with Q-VD-OPh treatment had no effect on AIF translocation (Figure 3C,D).

Q-VD-OPh Inhibited Caspase-3 Activation in Both Female and Male Mice
To assess the effect of cytochrome C activation on caspase activation, we detected the expression of caspase-3, caspase-8, and caspase-9 with Western blots. Expression of active (cleaved) caspase-3 was upregulated in the cytosol after stroke in both male and female mice. Q-VD-OPh inhibited caspase-3 cleavage in both female and male mice after stroke (Figure 4A,B). There were no differences in caspase-8 and caspase-9 expression in the cytosol after stroke (data not shown).

Discussion
The present study demonstrates several important findings. First, mitochondrial cytochrome C release, a major triggering

Table 1. Behavioral Scores After Stroke in Low (A) and High (B) Doses Q-VD-OPh Treated Mice

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Young mice were administered either 5 mg/kg or 20 mg/kg Q-VD-OPh, and aging mice were administered 10 mg/kg. There was a significant difference between behavioral scores in young and aging female drug-treated mice at 1.5 hours and 6 hours or 48 hours after stroke. *P < 0.05 compared with 6 hours and 48 hours in the same column; 1-way ANOVA.

A indicates aging; E, 17β-estradiol; F, female; O, oil; Ovx, ovariectomized; Q, Q-VD-OPh; V, vehicle.

No differences were seen in variables between vehicle- and drug-treated mice of either sex before (not shown) and 60 minutes after MCAO. No differences were seen in aging compared to young mice (not shown).

LDF indicates laser Doppler flowmetry; MAP, mean arterial pressure.

Table 2. Physiological Measurements in Vehicle- and Drug-Treated Mice

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No differences were seen in variables between vehicle- and drug-treated mice of either sex before (not shown) and 60 minutes after MCAO. No differences were seen in aging compared to young mice (not shown).
event for subsequent caspase activation, is higher in female mice soon after an induced stroke. Second, there are sex differences in the timing and duration of caspase activation after ischemia. Cleaved caspase-8 levels increase in the nucleus in both sexes after MCAO, but this predominates in females. Caspase-3 cleavage occurs in both sexes after MCAO, although this is more pronounced in the female brain. To evaluate the functional significance of the enhanced cytochrome C-caspase activation in females, we administered a pan-caspase inhibitor to mice of both sexes. Q-VD-OPh was neuroprotective in females but had no effect in males. Activation of caspase-3 was reduced in both sexes after Q-VD-OPh treatment; this effect was more robust in females. Third, treatment with Q-VD-OPh had no effect on ischemia-induced nuclear AIF translocation in either sex, demonstrating the relative specificity of this agent for inhibiting caspase-mediated cell death. Fourth, the sexually dimorphic protective effect of Q-VD-OPh was seen in intact females, Ovx females, and estrogen-replaced females, suggesting that its neuroprotective effect is independent of estrogen. Finally, because caspase-mediated cell death may play a more important role in developmental cell death, we administered Q-VD-OPh to aged mice of both sexes. Aging females continued to have a dramatic neuroprotective response to caspase inhibition. Similar to our results in young males, we found no protective response in aging male mice.

Over the past several years, data have been emerging that suggest that cell death mechanisms are sexually dimorphic. In a study using cytotoxic agents to induce cell death, female neurons demonstrated greater resistance to nitrosative stress than male neurons, yet had higher levels of cytosolic cytochrome C, an initiating event in the intrinsic caspase cascade. Male and female neurons also responded differently to drugs targeting specific proteins and pathways. In cultured neurons, female-derived neurons were differentially protected by z-VAD.fmk, a pan-caspase inhibitor. In sex-selected hippocampal slices exposed to oxygen-glucose deprivation, male-derived slices were protected with neuronal NO synthase inhibitors, and female slices were not. This suggests that female neurons may be differentially sensitive to caspase-induced cell death, and male neurons may be differentially sensitive to nitrosative cell death.

Similar findings have been seen in vivo. Enhanced caspase-3 activation is present in neonatal females compared to males after a hypoxic-ischemic insult. In the immature brain, male neurons displayed a more pronounced translocation of AIF than females after hypoxic ischemic. Surprisingly, we saw no sex differences in nuclear AIF translocation after stroke (Figure 3C,D); this may be attributable to differences in developmental age or the ischemic model used. Q-VD-OPh had no effect on stroke-induced AIF translocation in either sex. The caspase-independent AIF-mediated cell death pathway may not be a major contributor to cell death in females because the continued translocation of AIF did not counteract the protective effect of Q-VD-OPh. In contrast, males showed no neuroprotective effect of Q-VD-OPh treatment, despite reductions in caspase-3, suggesting that caspase-induced cell death does not predominant in males. Caspases play an essential role during apoptotic cell death and are synthesized as inactive zymogens that are proteolytically processed to produce mature active proteases. The involvement of caspases in stroke has been inferred from...
studies demonstrating that caspase inhibitors are neuroprotective in animal models of ischemia, as is genetic deletion of caspase-3. However, the literature examining the neuroprotective effects of caspase inhibition is mixed, with some showing protection and others not. Often, the sex of the animals is not mentioned, which could lead to significant variability.

Q-Vd-OPh is protective in neonatal hypoxic ischemic models, but only in females, consistent with our findings. In adult mice, females had an early robust release of cytochrome C at 6 hours (Figure 3A, band 3), which was attenuated with Q-Vd-OPh treatment. In contrast, males had the most robust increase at 12 hours after stroke (Figure 3A, band 12), which was not reduced by caspase inhibition. At this relatively late time point after stroke, it is likely that other parallel cell death pathways are activated in males (ie, PARP/AIF), which could lead to the enhanced mitochondrial damage and cytochrome C release seen at these later time points. It is possible that treatment with Q-Vd-OPh, by blocking caspase activation, leads to enhanced flux through the deleterious PARP/AIF pathway that predominates in males.

We examined caspase-3 in our studies because it is a major downstream executioner caspase. Our results revealed that the expression of cleaved caspase-3 increased 6 hours after stroke, which was attenuated by Q-Vd-OPh, with a more robust effect in females (Figure 4A,B), consistent with the early release of cytochrome C (Figure 3A at 6 hours). Q-Vd-OPh exerts its inhibitory function by forming an irreversible thioether bond between its aspartic acid derivation and the active site cysteine of the caspase. After stroke, caspase-9 is activated by the apoptosome and activates caspase-3. Q-Vd-OPh treatment decreased caspase-3 activation after stroke below the levels seen in male and female sham mice. It is possible that ischemic insults activate the active binding site at cysteine more robustly so that Q-Vd-OPh binds more strongly to the site. Alternatively, caspase-3 may be randomly activated by other pathways in surgical shams that are independent of that mediated by Q-Vd-OPh. Striking sex differences were present in the nuclear activation of caspase-8, with females having a dramatic increase in cleavage products compared to males after stroke (Figure 1). Caspase-8 is the primary activator of the extrinsic death receptor pathways of apoptosis. However, caspase-8 can also induce apoptosis through the intrinsic mitochondrial pathway by cleaving the cytoplasmic factor Bid, a proapoptotic member of the Bcl-2 family. Nuclear translocation of caspase-8 is known to occur after stroke in males, where it cleaves and inactivates PARP-2. Deletion of PARP-2 is protective in male mice; therefore, the reduction in PARP-2 activity by caspase-8 could be beneficial in stroke. However, we have previously shown that deletion of the major isoform of PARP, PARP-1, is detrimental in female animals. The role of PARP-2 in female ischemic cell death has not yet been investigated; however, because nuclear levels of active caspase-8 are higher in females after stroke, this could lead to a differential reduction in PARP-2 activation, which is analogous to the loss of PARP-1 and could lead to an exacerbation of injury in females.

As expected, intact female and Ovx plus E2-treated female mice had smaller histological damage than Ovx plus oil-treated female mice in our vehicle-treated groups (Figure 2C). Q-Vd-OPh maintained its protective effect in all female groups, including aging mice (Table 2; Figure 2C,D). Because the majority of stroke deaths in the United States now occur in women who are postmenopausal, this is an important point if these agents are developed for clinical use. Aging female mice had larger infarct volumes than young female mice after stroke, likely secondary to the loss of estrogen with aging (Figure 2A,B). Aging males had significantly smaller strokes compared to young males (total: aging: 30.7±10.8%, n=6 vs young: 54.6±2.0%, n=6; P<0.01; Figure 2C,D). In previous studies, both hypertension and diabetes increased stroke volume, but age did not (in 3-, 9-, and 20-month-old rats). Despite this lack of histological effects of aging on infarct volume, invariably significantly higher mortality rates and more severe neurological impairments are found in the older animals, as was seen in these studies. Also in agreement with our findings, others have found that histological infarct damage is paradoxically higher in young (3 months) compared to old (24 to 26 months) male rats. Interestingly, a recent study that found an increase in infarct size and enhanced blood–brain barrier disruption in aged rats used only female animals. The enhanced injury may reflect the loss of endogenous estrogen rather than an effect of aging per se. This finding is also consistent with our data, which demonstrated enhanced MCAO-induced injury in aging female mice, and again emphasizes the importance of examining both sexes in experimental stroke studies.

In conclusion, we have shown sex differences in stroke outcomes after administration of the broad-spectrum caspase inhibitor Q-Vd-OPh. Female mice have improved neurological deficits and decreased infarct volumes, whereas males do not. Females have early release of cytochrome C and caspase activation after stroke. These data provide evidence for parallel sex-dependent cell death pathways in the ischemic brain. These sex differences need to be further explored if we are to develop efficacious neuroprotective agents for use in stroke patients.

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**Disclosures**

None.

**References**


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