Sex Differences in the Response to Poly(ADP-ribose) Polymerase-1 Deletion and Caspase Inhibition After Stroke

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Background and Purpose—Emerging data suggest that the molecular cell death pathways triggered by ischemic insults differ in the male and female brain. Cell death in males is initiated by poly(ADP-ribose) polymerase-1 (PARP-1) activation; however, manipulation of this pathway paradoxically increases ischemic damage in females. In contrast, females are exquisitely sensitive to caspase-mediated cell death. The effect of caspase inhibition in PARP-1 knockout mice was evaluated to determine if the detrimental effects of PARP deletion in females were secondary to increased caspase activation.

Methods—-Focal stroke was induced by transient or permanent middle cerebral artery occlusion (MCAO) in wild-type (WT) and PARP-$1^{-/-}$ mice of both sexes. The pan-caspase inhibitor, quinoline-Val-Asp(Ome)-CH2-O-phenoxy (Q-VD-OPh), was administered 90 minutes after middle cerebral artery occlusion. Infarct size and neurological sores were assessed. Separate cohorts were used for protein analysis for PAR, Apoptosis inducing factor (AIF), caspase-9, and caspase-3.

Results—WT mice of both sexes had increased nuclear AIF after stroke compared to PARP-$1^{-/-}$ mice. PARP-$1^{-/-}$ females had higher mitochondrial cytochrome C and activated caspase-9 and -3 levels than WT female mice. PARP-$1^{-/-}$ females also had an increase in stroke-induced cytosolic cytochrome C release compared with WT females, which was not seen in males. Q-VD-OPh decreased caspase-9 in both males and females but only led to reduction of infarct in females. PARP-$1^{-/-}$ males had smaller infarcts, whereas PARP-$1^{-/-}$ females had larger strokes compared with WT. Q-VD-OPh significantly decreased infarct in both WT and PARP-$1^{-/-}$ females in both transient and permanent MCAO models, but had no effect in males.

Conclusions—Deletion of PARP-1 reduces infarct in males but exacerbates injury in females. PARP-$1^{-/-}$ females have enhanced caspase activation. The detrimental effects of PARP loss in females can be reversed with caspase inhibition. **(*Stroke*. 2011;42:1090-1096.)**

Key Words: AIF ■ caspase ■ MCAO ■ PARP ■ sex differences

It is increasingly recognized that stroke is a sexually dimorphic disease both experimentally and clinically, although these sex differences are still poorly understood.[1] Although estrogen has long been suggested to contribute to sex differences in stroke,[2] nonhormonal factors are also involved in ischemic sexual dimorphism.[3] Accumulating experimental studies have demonstrated sex differences in ischemic cell death in vitro from cultures derived from male versus female cells.[4,5] Caspase inhibition dramatically reduced injury in female but not male pups,[6] whereas poly(ADP-ribose) polymerase (PARP) deletion protected male pups but not females.[7] We have previously reported similar findings in adult stroke models.[8–10] Importantly, in these previous studies, PARP inhibition or deletion significantly exacerbated injury in females, suggesting that PARP activation may be protective in the female brain.

In this study, we tested the hypothesis that loss of PARP leads to upregulation of caspase-mediated pathways in PARP-1 knockout (KO) females, leading to elevated levels of caspases and subsequent enhancement of damage after middle cerebral artery occlusion (MCAO). We administered quinoline-Val-Asp(Ome)-CH2-O-phenoxy (Q-VD-OPh), a novel broad-spectrum caspase inhibitor, to determine if the detrimental effects of PARP deletion in females could be ameliorated with caspase inhibition.

Materials and Methods

Animals

PARP-1 KO and corresponding wild-type (WT; SV-129 Brown) mice were originally purchased from Jackson laboratories (KO) or Taconic (WT; Germantown, NY) and bred in-house (F9). All experiments were performed according to National Institutes of Health guidelines for the care and use of animals in research and

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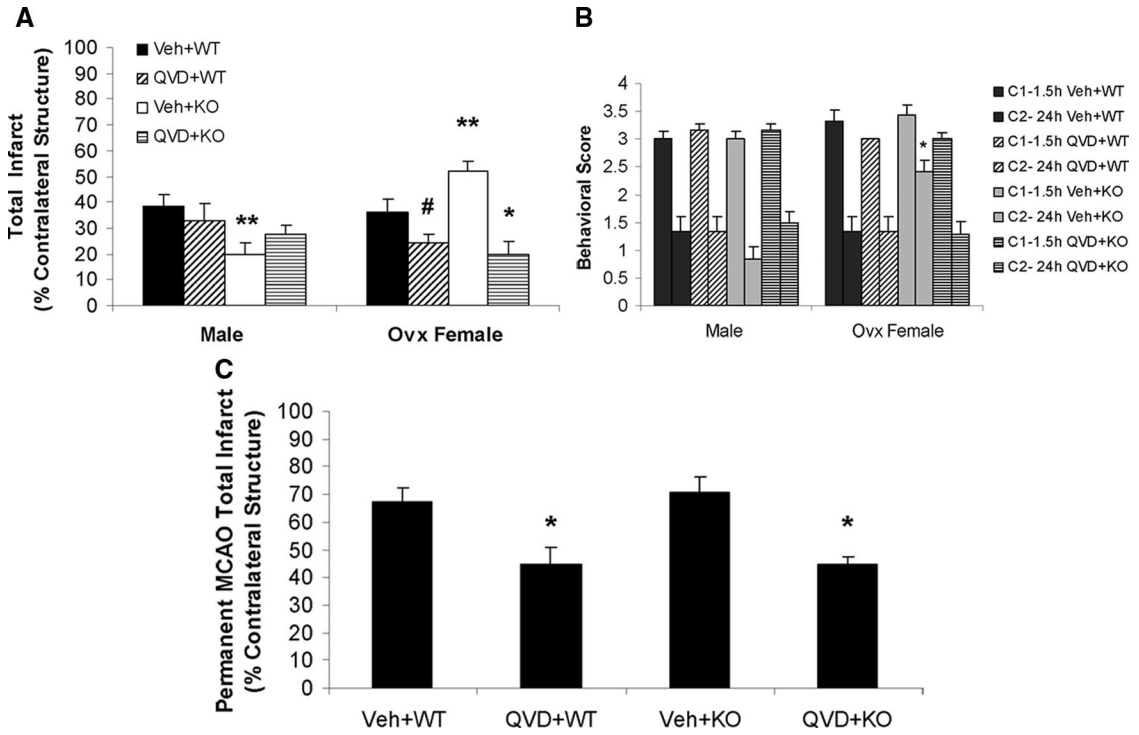

Figure 1. Stroke outcomes in male and female mice after MCAO. A, Total infarct (24 hours after tMCAO); percentage of contralateral structure. *$P<0.01$ vs vehicle-treated KO female group; **$P<0.05$ vs vehicle-treated WT mice; #$P<0.05$ vs vehicle-treated WT female group. B, Neurological deficits at 1.5 hours and 24 hours of MCAO. The adjacent columns (C1 and C2) of the same pattern indicate the same group at the 1.5 hours and 24 hours time points. *$P>0.05$ vs 1.5 hours in vehicle-treated KO female group, and *$P<0.05$ vs 24 hours in other female groups. C, Total infarct after pMCAO. Brains were assessed at 48 hours of stroke. *$P<0.05$ vs vehicle-treated group. Veh indicates vehicle; QVD, Q-VD-OPh; WT, wild-type; KO, knockout; C, column.

under protocols approved by the University of Connecticut Health Center Animal Care and Use Committee. Young mice (9 to 12 weeks; 21 to 25 g) of both sexes were used. Genotypes were confirmed with polymerase chain reaction.

Ovariectomy and Hormone Measurements

To determine the contribution of estrogen and to normalize infarct size, mice of each genotype were ovariectomized 2 weeks before MCAO. Loss of estrogen was confirmed by enzyme-linked immunosorbent assay for serum 17β-estradiol levels (IBL HAMBURG, Hamburg, Germany) and uterine weights as previously described.[11]

Focal Cerebral Ischemia

Focal transient cerebral ischemia was induced by MCAO (tMCAO) for 90 minutes followed by reperfusion as described previously.[12] Permanent MCAO (pMCAO) was induced in a similar fashion, but the occluding suture remained in the middle cerebral artery until euthanasia (48 hours after stroke). Cerebral blood flow was measured by laser Doppler flowmetry (Moor Instruments Ltd) during MCAO. Neurological deficits were scored at 1.5 hours and 24 hours poststroke, as described in Liu et al.[12]

Drug Administration

Q-VD-OPh (MP Biomedicals, Aurora, OH; 25 mg/kg) was dissolved in dimethyl sulfoxide and further diluted with sterile phosphate-buffered saline (10% dimethyl sulfoxide) and injected intraperitoneally (0.21 to 0.25 mL total volume based on body weight) at reperfusion or 90 minutes postocclusion as in Liu et al[8] in both stroke and surgical shams. Control mice were injected with vehicle alone.

Histological Assessment

Twenty-four hours after stroke, the mice were euthanized and the brains were stained with 1.5% 2,3,5-triphenyltetrazolium solution as previously described.[12]

Subcellular Fractionation and Western Blotting

Brain samples were obtained by rapid removal of the brain 6 hours after MCAO and were fractionated into the cytosol, mitochondrial, and nuclear fraction as described in Liu et al.[8] Protein concentration was determined by a BCA Protein Assay Kit (Thermo Fisher Scientific Inc, Rockford, IL) and subjected to Western blotting as described.[11] Primary antibodies were: PAR polymers (BD Biosciences; 1:1000), AIF (Abcam; 1:500), caspase 9 (Cell Signaling; 1:1000) and -3 (Santa Cruz Biotechnology; 1:500), macrophage migration inhibitory factor (MIF; 1:1000; Cell Sciences), COX IV (Abcam; 1:1000), and Histone H3 (MIF; 1:4000; Abcam). Secondary antibodies were from Santa Cruz: goat antirabbit IgG 1:5000, goat antimouse IgG 1:2000, and donkey antigoat IgG 1:1000. Densitometry of Western blots was analyzed with Scion Image as described in Liu et al.[11]

Statistics

Data from individual experiments were presented as mean±SEM and analyzed with a *t* test for 2 groups and 2-way analysis of variance (with Turkey post hoc correction for multiple comparisons where appropriate) for the comparison of the means between the experimental groups. Neurological deficit scores were analyzed with the Mann–Whitney *U* test. $P<0.05$ was considered statistically significant. All behavioral and histological assessments were performed by an investigator blinded to genotype/drug treatment.

Results

PARP-1 Deletion Protected Males But Exacerbated Injury in Females After MCAO

PARP-$1^{-/-}$ male mice had significantly smaller infarct than WT male mice (KO versus WT: 19.7%±4.5% versus 38.4%±3.8%; n=9/group, $P<0.05$; Figure 1A), confirming

Table. Physiological Measurements in Vehicle- and Drug-Treated KO Mice*

Group	pH	pO_2, mm Hg	pCO_2, mm Hg	Glucose, mg/dL	MABP, mm Hg	LDF, % Ischemia	LDF, % Reperfusion
Male							
Vehicle	7.34±0.12	86±5	38±11	170±17	61±3	11±1	87±4
Drug	7.28±0.07	88±13	31±9	166±26	65±4	11±3	90±3
Female							
Vehicle	7.29±0.07	94±11	39±5	181±16	63±3	12±1	90±2
Drug	7.26±0.13	91±18	34±11	178±29	63±4	11±2	89±3

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

MABP indicates mean arterial blood pressure; LDF, laser Doppler flowmetry.

the protective effect of PARP deletion in males in this model.[10] PARP-$1^{-/-}$ ovariectomized female mice exhibited a significant exacerbation in infarct size compared with WT ovariectomized females (KO versus WT: 52.2%±4.8% versus 36.0%±4.5%; n=12/group, $P<0.05$).

Q-VD-OPh Treatment Was Neuroprotective in Female But Not Male WT and PARP-$1^{-/-}$ Mice

Administration of Q-VD-OPh had no effect on infarct in male WT mice; although there was an apparent increase in damage in PARP-$1^{-/-}$ mice treated with Q-VD-OPh, this trend did not reach statistical significance (Figure 1A). In females, treatment with Q-VD-OPh significantly decreased infarct volumes in WT ovariectomized mice (vehicle versus drug: 36.0%±4.5% versus 24.1%±3.5%; n=12/group, $P<0.05$). Q-VD-OPh administration significantly reduced infarct size in PARP-$1^{-/-}$ female mice (vehicle versus drug: 52.2%±4.8% versus 19.9%±5.2%; n=12/group, $P<0.01$) to levels seen in WT Q-VD-OPh-treated mice. All cohorts had improved behavioral deficits at 24 hours compared with that seen immediately (1.5 hours) after stroke except PARP-$1^{-/-}$ vehicle-treated females, which had worse deficits at 24 hours (Figure 1B). PARP-$1^{-/-}$ males had lower scores than WT males, and PARP-$1^{-/-}$ females had higher scores than WT, an effect that was reversed with Q-VD-OPh treatment.

Q-VD-OPh Decreased Infarct Volumes in Ovariectomized Females Subjected to pMCAO

To test whether the effect of Q-VD-OPh on stroke was model-dependent, or dissipated at longer survival end points, another cohort of ovariectomized WT and PARP-1 KO female mice were subjected to MCAO with 48 hours survival. Similar to the results seen in temporary/reperfusion models, damage from pMCAO was exacerbated in KO compared with WT littermates. Infarct was more severe in the permanent model in both genotypes compared with transient ischemia models in vehicle-treated groups: WT pMCAO versus tMCAO: 64.3%±5.0% versus 36.0%±4.5%, n=7/group, $P<0.01$; KO pMCAO versus tMCAO: 74.86%±5.2% (n=6) versus 52.2%±4.8% (n=12), $P<0.05$ (Figures 1A, C). Q-VD-OPh treatment reduced infarct damage after pMCAO in both WT and PARP-1 KO female mice (Figure 1C).

Physiological Parameters, Serum Estrogen Levels, and Uterine Weights Were Not Significantly Different Between WT and KO Female Mice

No differences in pH, pO_2, pCO_2, blood glucose, mean arterial pressure, or laser Doppler flowmetry were seen between vehicle- and drug-treated mice of either WT (data not shown) or PARP-$1^{-/-}$ mice (Table). There were no differences in estrogen levels between any of the animal groups (Figure 2A). All female mice had low uterine weights and atrophy (Figure 2B) consistent with the loss of end-organ estrogenic effects.[11]

PARP-1 Deletion Significantly Reduced PAR Polymer Formation and AIF Translocation in Both Male and Female Mice

PAR polymer formation and nuclear AIF translocation 6 hours after tMCAO were examined. In both male and female WT mice, nuclear PAR levels dramatically increased after stroke compared with sham; however, there was no difference

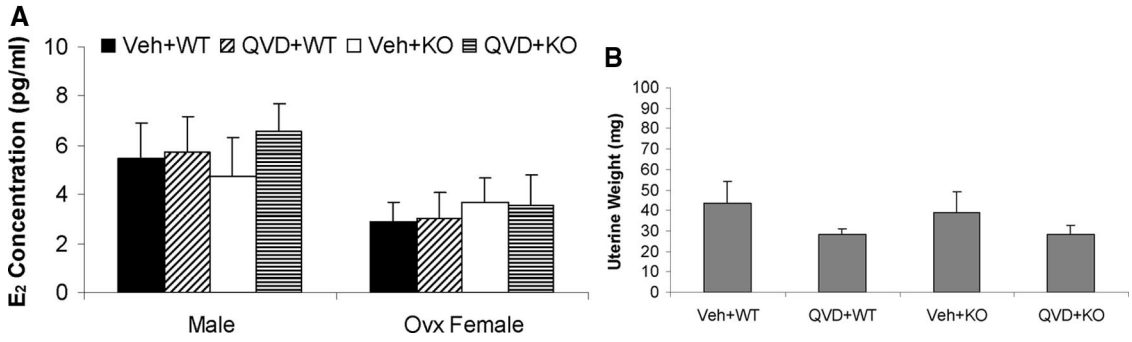

Figure 2. Serum estrogen levels (A) and uterine weights (B). No significant differences were found between groups. Veh indicates vehicle; QVD, Q-VD-OPh; WT, wild-type; KO, knockout.

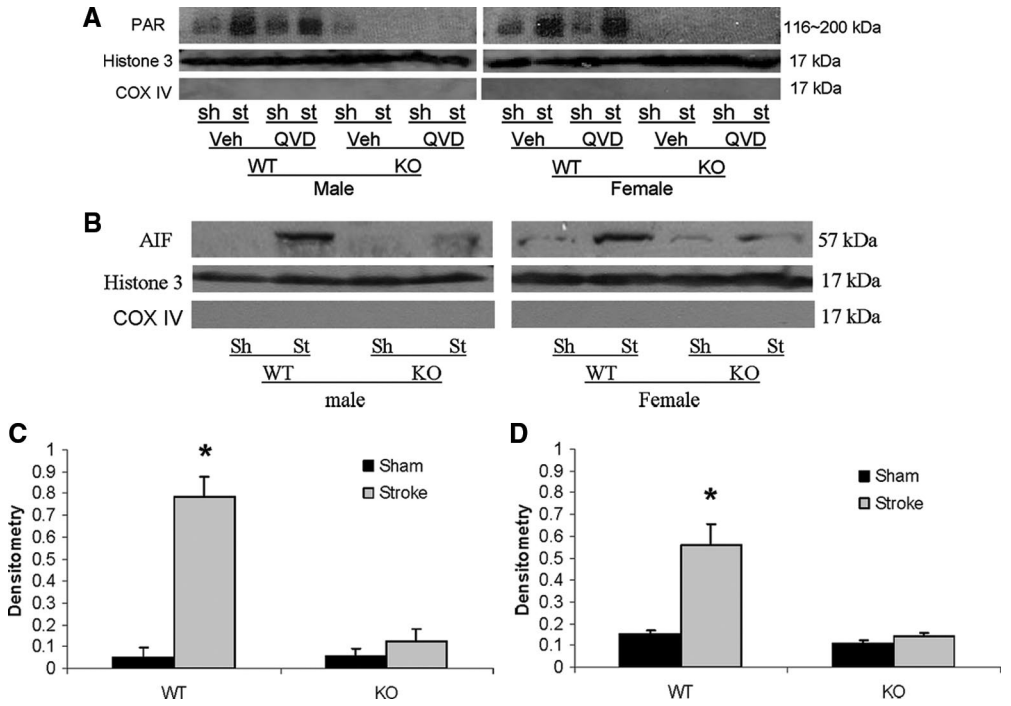

Figure 3. Western blots of key proteins in the PARP-1–AIF cell death pathway. A, Nuclear PAR in both male and female group (n=4 hemispheres/group). Each sample had biological triplicates. B, Nuclear AIF translocation in both male and female groups. n=6 animals/group, repeated in triplicate. Histone 3 served as the loading control and COX IV as the negative control to confirm nuclear fraction purity in both A and B. C–D, The optical density (OD) was expressed as the ratio of the AIF bands to control bands (histone 3). C, Male; D, female. *$P<0.01$ vs any other group. Veh indicates vehicle; QVD, Q-VD-OPh; WT, wild-type; KO, knockout; Sh, sham; St, stroke.

in PAR levels between vehicle- and drug-treated WT mice of either sex (Figure 3A; n=4/group). PARP-$1^{-/-}$ mice of both sexes had a dramatic reduction in nuclear PAR polymers as shown in Figure 3A regardless of Q-VD-OPh treatment. As expected, very little nuclear AIF translocation was seen in sham mice and KO mice (Figure 3B–D).

PARP-1 Deletion Had Different Effects on Cytochrome C and Caspase-3 Levels in Male and Female Mice After tMCAO

Mitochondrial cytochrome C and caspase-3 levels after tMCAO were higher in PARP-$1^{-/-}$ females compared with WT females, whereas no changes were seen as a consequence of either MCAO or PARP-1 deletion in male mice (Figure 4A–C). Cytosolic cytochrome C levels significantly increased after stroke in both WT and KO female mice; however, the ratio of sham to stroke-induced increase in cytosolic cytochrome C was significantly higher in PARP KO females compared with WT females. No differences in cytochrome C levels were seen in males (Figure 5A–C).

Q-VD-OPh Treatment Decreased Caspase-9 Levels in Female Mice After tMCAO

To assess the effect of Q-VD-OPh on caspase expression in PARP-$1^{-/-}$ mice, we administered drug or vehicle to PARP-$1^{-/-}$ male and female mice immediately after reperfusion. Activated (cleaved) caspase-9 levels were low in sham WT mice of both sexes (see Lane 1, Figure 6A). Stroke increased caspase-9 cleavage products in both female and male WT mice (Lane 2) with significantly higher levels in females (female: Lane 1 versus Lane 2; Figure 6B). Interestingly, PARP-$1^{-/-}$ females had higher levels of caspase-9 cleavage products both in sham and after MCAO compared with WT mice (female: Lanes 1 and 2 versus 3 and 4, respectively). Q-VD-OPh treatment reduced MCAO-induced caspase-9 activation in KO female and male mice after stroke (Lane 4 versus Lane 6) and reduced the baseline elevation in caspase activation in sham PARP-$1^{-/-}$ females (female: Lane 3 versus Lane 5). No differences in cleaved caspase-9 were observed between WT and KO vehicle-treated male mice (male: Lanes 1 and 2 versus Lanes 3 and 4, respectively; Figure 6C).

Discussion

This study examined PARP and caspase activation in cerebral ischemia. There are several important findings: (1) PARP-1 deletion is deleterious in females subjected to ischemic injury in both reperfusion[10] and permanent MCAO models; (2) caspase inhibition with Q-VD-OPh protects females but not males. Importantly, Q-VD-OPh decreased infarct volumes in both WT and KO female mice. Caspase inhibition reduced infarct to levels seen in WT Q-VD-OPh mice, rescuing the deleterious PARP KO phenotype. This neuroprotection was independent of acute estrogen levels because all the females were ovariectomized. In contrast, Q-VD-OPh had no effect in either WT or KO males. Taken together, this implies that the effect of caspase inhibition is dependent on sex but independent of PARP; (3) the neuroprotective effect of caspase inhibition was seen in a permanent ischemia model and was present even after 48 hours. In this model, the differences between WT and PARP KO mice were more modest, likely due to a ceiling effect; (4) PAR polymer formation and nuclear AIF translocation were increased after stroke in both

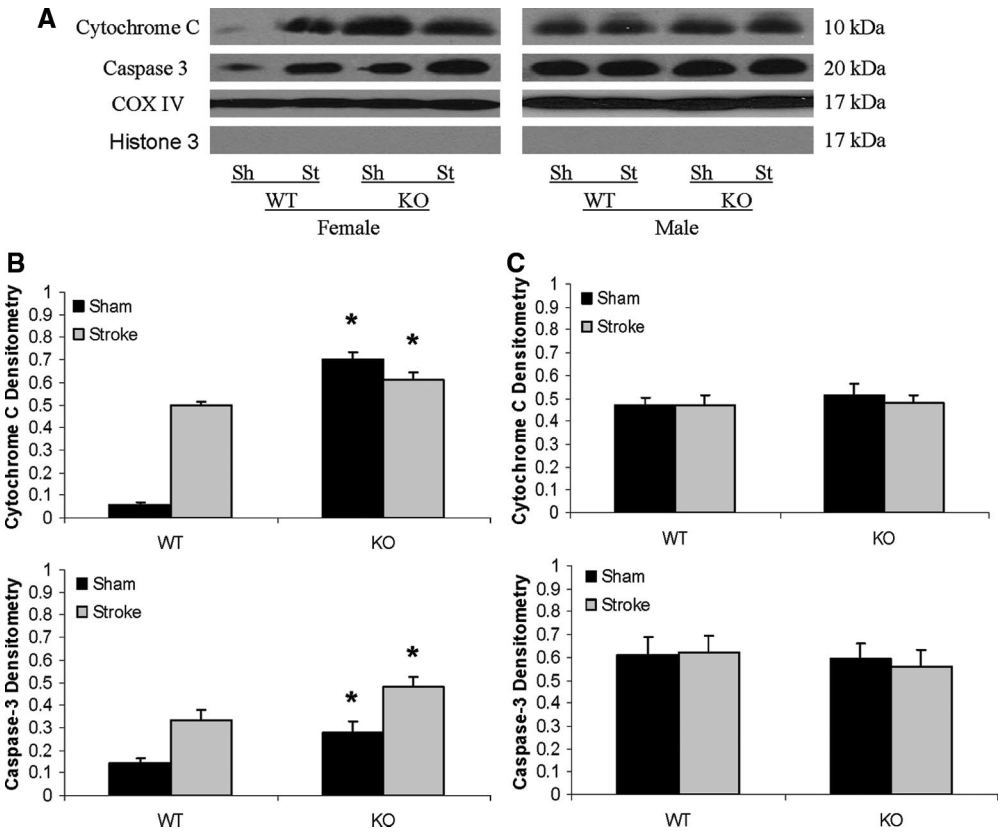

Figure 4. Western blots of key proteins in cytochrome C-caspase cell death pathway in the mitochondrion. A, Cytochrome C and caspase-3 in both male and female groups. COX IV served as the loading control and histone 3 as the negative control to confirm the purity of the mitochondrion. B–C, OD was expressed as the ratio of the cytochrome C (upper panel) or caspase-3 (lower panel) bands to control bands. B, Female; (C) male. *$P<0.05$ vs WT counterparts. n=6 animals/group, samples run in triplicate. Veh indicates vehicle; QVD, Q-VD-OPh; WT, wild-type; KO, knockout; Sh, sham; St, stroke.

male and female WT mice. PARP-1 deletion attenuated both PAR formation and nuclear AIF translocation, but this led to different effects depending on the sex of the animal, with neuroprotection in males and increased infarct in females; (5) PARP-$1^{-/-}$ females had a baseline elevation in mitochondrial cytochrome C and activated caspase-3 and -9 expression, suggesting that the brain is "primed" to turn on caspase pathway, and does so even without an identified stressor, for example, ischemia; and (6) stroke induced significantly more cytosolic cytochrome C release and caspase activation (as measured by cleavage products) in PARP-1 KO females compared with either WT females or KO males, showing a further response to this "priming." Q-VD-OPh ameliorated this abnormal caspase response in KO female mice.

Emerging data provide evidence for striking and previously unsuspected sexual dimorphism in cell death pathways induced by ischemic injury and this study provides further evidence for this concept. In experimental models, males preferentially use caspase-independent mechanisms to trigger cell death initiated by PARP activation with subsequent nuclear AIF translocation.[9,10,13,14] The smaller infarcts in PARP KO males support the hypothesis that PARP-1 is an important mediator of cell death in male animals.[15] Interestingly, these same molecular events occur in females[10] (Figure 3), but do not lead to cell death as evidenced by PARP KO females who have significant reductions in PAR and AIF yet have an exacerbation in stroke damage compared with WT. The significant exacerbation in damage in PARP-1 KO females after MCAO suggested that PARP activation is either (1) a protective in females; or (2) that loss of PARP allows for activation of a more deleterious pathway. Because female neurons,[5] neonates,[6] and adults[8] are more sensitive to caspase-mediated cell death, we first evaluated caspase activation in PARP KO mice. PARP-$1^{-/-}$ females had higher levels of mitochondrial cytochrome C, caspase-9, and caspase-3 than WT mice suggesting that the caspase pathway is induced by the loss of PARP. The importance of changes in mitochondrial levels of cytochrome C is unknown. Traditionally, the triggering factor for caspase-mediated cell death is the mitochondrial release of cytochrome C into the cytosol[16]; however, no previous studies examined what occurs in the female brain in the mitochondrial fraction. WT females have increased cytosolic cytochrome C release with injury compared with males,[8] and the timing of release differs by sex in neonatal models.[6] In this study, we saw an increase in cytosolic cytochrome C levels after stroke in both WT and PARP-$1^{-/-}$ females versus sham females. No changes were seen in males with stroke at this time point. Importantly, in agreement with our hypothesis that PARP females have enhanced caspase-induced cell death due to the loss of PARP, PARP KO females had significantly more cytosolic cytochrome C release than WT females (expressed as a ration of stroke/sham).

Controversy exists in the literature as to the effects of Q-VD-OPh on male animals.[6,17,18] In this study Q-VD-OPh

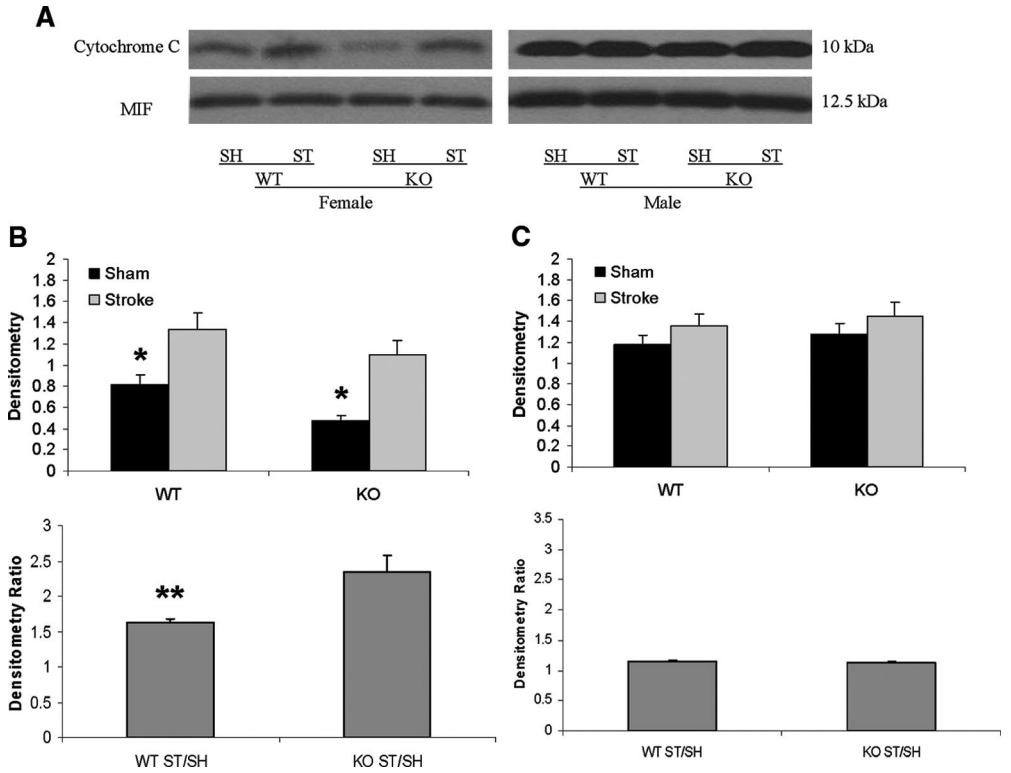

Figure 5. Western blots of cytochrome C in the cytosol. A, Cytochrome C levels in both male and female group. MIF served as the loading control. B–C, OD (upper panel) was expressed as the ratio of the cytochrome C bands to control bands. The density ratio (lower panel) was calculated by dividing densitometry of stroke group with that of the sham group. (B) Female; (C) male. *$P<0.05$ vs stroke group; **$P<0.05$ vs KO group. n=6 animals/group, samples run in triplicate. Veh indicates vehicle; QVD, Q-VD-OPh; WT, wild-type; KO, knockout; Sh, sham; St, stroke.

attenuated the protective effect of PARP-1 deletion in males (Figure 1A), suggesting that caspase activation may either be a protective response in the male brain or alternatively that males are insensitive to downstream events triggered by caspase activation. This could account, in part, for the variable results seen in preclinical stroke models that have examined the effects of caspase inhibition, the vast majority of which were performed exclusively in male animals.[19]

Levels of cytochrome C, activated caspase-9, and caspase-3 were significantly higher in both sham and MCAO PARP KO females compared with sex-matched WT. This interaction between caspase-dependent and -independent pathways has not been previously reported and could explain the severe phenotype seen in PARP-$1^{-/-}$ females after MCAO. The translational importance of these findings becomes clear when one considers the pharmacological agents currently being developed for clin-

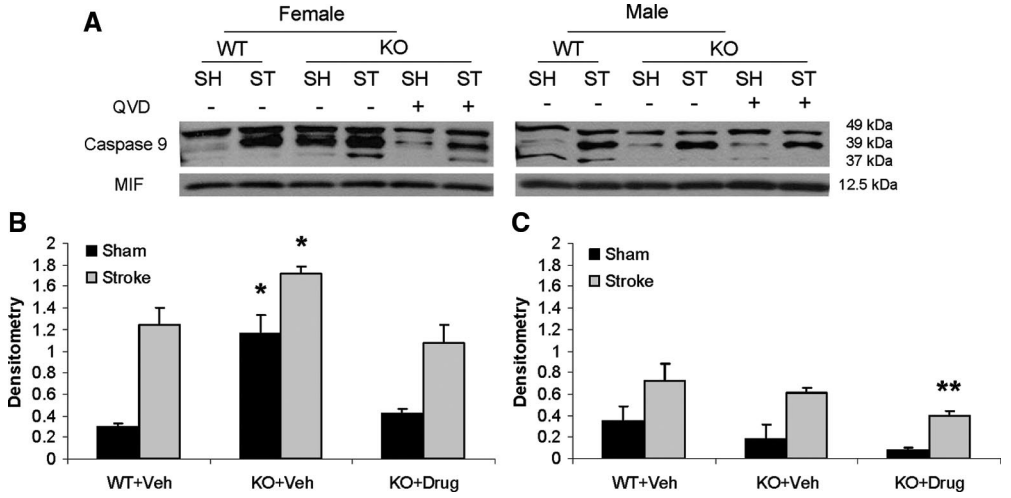

Figure 6. Q-VD-OPh treatment decreases expression of activated Caspase-9 in females. A, Caspase-9 levels in the cytosol with (+) or without (vehicle −) Q-VD-OPh treatment. MIF served as the loading control. B–C, OD was expressed as the ratio of cleaved caspase-9 bands (39 and 37 kDa) to control bands. *$P<0.05$ vs WT and drug-treated KO counterparts; **$P<0.05$ vs vehicle-treated KO counterpart. n=6 animals/group, samples run in triplicate. Veh indicates vehicle; QVD, Q-VD-OPh; WT, wild-type; KO, knockout; Sh, sham; St, stroke.

ical trials such as minocycline, which acts as a PARP inhibitor[20] and has sex-specific effects in preclinical models.

Suggesting that an absolute dichotomy exists in sensitivity to specific cell death pathways is overly simplistic. These 2 cell death pathways do not act independently, because cross-talk exists through the interaction of AIF and cytochrome C in the mitochondrion[21] and PARP can be directly cleaved by caspase-3 and -7.[22] PARP activation enhances formation of PAR polymers that are directly toxic when injected into neurons, leading to apoptosis and nuclear condensation inducing cell death even without loss of $NAD.^{+}$ [23] The downstream nuclear events that trigger the final phase of cell death in females remain unknown.

There are several limitations to this work, and our results must be interpreted with these in mind. Stroke-induced changes in caspases and cytochrome C in the mitochondria need to be confirmed. Despite appropriate loading controls, technical issues (ie, cytosolic contamination) could be a factor. We only evaluated a genetic model of PARP deficiency, which has had the loss of PARP-1 throughout development. Findings could differ in pharmacological models, because the enhanced baseline and stroke-induced caspase activation may take time to develop. We specifically did not use PARP-1 inhibitors secondary to possible interactions with Q-VD-OPh dosing and metabolism. We only examined ovariectomized female mice in this study for several reasons: (1) the ovariectomy model is more translationally relevant to the population at risk for stroke, postmenopausal women; (2) stroke outcomes are more variable in gonadally intact females due to fluctuating levels of estrogen across estrus[24]; and (3) we were specifically interested in examining sex rather than hormone effects; therefore, hormone replacement was not used. We recognize that acute ovariectomy cannot ameliorate the many developmental "organizational" effects of steroids,[25] and the contribution of ovarian steroids versus sex (XX versus XY) will be evaluated in future studies using genetic models.

In conclusion, PARP-1 deletion and caspase inhibition have strikingly different effects on stroke outcomes that are dependent on the sex of the animal evaluated. Sensitivity to caspase activation predominates in females, whereas stroke-induced cell death in males is mediated by PARP-1–AIF activation. Removal of PARP-1 protects males but leads to a paradoxical exacerbation of injury in females. This is secondary to enhancement of caspases and can be reversed with caspase inhibition. Understanding the sex-specific sensitivity to cell death has important translational relevance for the development of neuroprotective agents.

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Disclosures

None.

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