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Sex Differences in the Response to Activation of the Poly (ADP-ribose) Polymerase Pathway after Experimental Stroke

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Abstract

It is increasingly recognized that histological and functional outcomes after stroke are shaped by biologic sex. Emerging data suggests that ischemic cell death pathways are sexually dimorphic (Hurn et al., 2005; Lang and McCullough, 2008). Reducing neuronal nitric oxide (NO) or poly-ADP ribose polymerase (PARP-1) activation protects only the male brain (Hagberg et al., 2004), and paradoxically enhances ischemic injury in females (McCullough et al., 2005). In this study, we examined downstream mediators of NO/PARP activation to investigate possible mediators of ischemic sexual dimorphism. Nuclear translocation of Apoptosis Inducing Factor (AIF) was equivalent in wild-type males and females after stroke and was unaffected by estrogen exposure. Deletion of PARP1 led to a dramatic reduction in stroke-induced poly(ADP-ribose) polymerase (PAR) formation and AIF translocation in both sexes, yet ischemic damage was reduced only in males. Subsequent examination of AIF-deficient Harlequin mice demonstrated that male Harlequin mice had less PAR formation, reduced AIF translocation and less ischemic damage than male wild-type mice. In contrast, female Harlequin mice had no neuroprotective effect of gene deletion despite robust reductions in PAR formation and AIF translocation. Although equivalent activation of this cell death pathway occurs in both sexes after ischemia, detrimental effects are only present in males. AIF translocation and PAR formation do not mediate ischemic injury in the female brain, therefore agents designed to reduce PARP1 activation are unlikely to benefit females.

Keywords

Stroke; Sex; Estrogen; Poly (ADP-ribose) Polymerase 1 (PARP1); Apoptosis Inducing Factor (AIF); Poly (ADP-ribose) Polymers (PAR); Middle Cerebral Artery Occlusion

Stroke affects 15 million people worldwide each year, and is the leading cause of long-term disability in the United States (Rosamond, et al., 2007). The epidemiology of ischemic stroke is sexually dimorphic in that ischemic events occur with greater frequency in men vs. women until advanced age (Sudlow and Warlow, 1997; Sacco et al., 2004; Incidence and Prevalence,

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2006). As the incidence of stroke increases dramatically in women after menopause, exposure to reproductive hormones may be a major contributor to this sex difference. Extensive preclinical evidence demonstrates that estrogen improves neuronal survival after injury both *in vivo* and *in vitro* (Garcia-Segura et al., 2001; McCullough and Hurn, 2003). However, recent clinical trials evaluating the efficacy of hormone replacement therapy in post-menopausal women for prevention of stroke (Rossouw et al., 2002; Wassetheil-Smoller et al., 2003) led to the surprising finding of increased vascular risk in hormone-treated women. In epidemiological studies, stroke rates do not climb until well after the menopause, suggesting that non-hormonal factors play a role in ischemic sexual dimorphism (Rosamond et al., 2007). In addition, sex differences in outcomes from stroke and hypoxic-ischemic encephalopathy are present in neonatal populations where levels of circulating hormones are equivalent between the sexes (Fullerton et al., 2003; Marlow et al., 2005; Zhu et al., 2006; Johnston and Hagberg, 2007, Renolleau et al., 2007).

Several distinct yet overlapping pathways contribute to cell death in experimental stroke. Mitochondrial damage results in the release of cytochrome C, triggering activation of the intrinsic pro-apoptotic caspase cascade and formation of the apoptosome (Ferrer and Planas 2003; Chan 2004; Stefanis, 2005). In addition, a distinct cell death pathway exists whereby DNA damage from rising levels of Nitric Oxide (NO) and peroxynitrite (ONOO) leads to the activation of the DNA repair enzyme poly ADP ribose polymerase-1 (PARP-1) and formation of poly(ADP-ribose) polymerase (PAR) polymers. This triggers the release of apoptosis inducing factor (AIF) from the mitochondria leading to *caspase-independent* cell death (Bredt et al., 1990; Susin et al., 2000; Yu et al., 2002). Key evidence in establishing NO toxicity/PARP-1 activation as a major cytotoxic mechanism has accumulated from exclusively male animals or mixed sex primary neuronal cell cultures (Huang et al., 1994; Eliasson et al., 1997; Cao et al., 2003; Wang et al., 2004). In contrast, we have recently discovered that this pathway exhibits dramatic sexual dimorphism and that the integrity of nNOS/PARP-1 signaling is paradoxically protective in the female (McCullough et al., 2005). Similar sex disparities exist *in vitro*, as female neurons are intrinsically protected after an oxidative challenge, eliminating differences in gonadal hormone levels as the sole explanation for these findings (Du et al., 2004; Li et al., 2005).

We propose that there are distinct sex-based cell death programs that involve the differential regulation of the NO/PARP pathway and its downstream mediator AIF. This pathway mediates cell death in the male, but not the female brain. We utilized PARP deficient and low AIF-expressing mice to determine if reductions in PARP/AIF activation would protect the female brain.

Methods

Experimental Animals and groups

PARP1 $-/-$, Harlequin (HQ) mutant mice expressing low levels of AIF (*Pdcd8^{Hq}; Hq*) and wild type mice (WT) used in this study were purchased from The Jackson Laboratory (Bar Harbor, Me; Culmsee et al., 2005). The present study was conducted in accordance with the NIH guidelines for the care and use of animals in research and under protocols approved by the Animal Care and Use Committee of the University of Connecticut Health Center.

Ischemic model

Cerebral ischemia was induced by 90 min of reversible middle cerebral artery occlusion (MCAO, 20–25 gm mice 10–12 weeks of age) under Isoflurane anesthesia as previously described (McCullough et al., 2005). Rectal muscle temperatures were maintained at approximately 37°C during surgery and ischemia with an automated temperature control

feedback system. A midline ventral neck incision was made, and unilateral MCAO was performed by inserting a 6.0 Doccol monofilament (Doccol Corp, Redland, CA) into the right internal carotid artery 6 mm from the internal carotid/pterygopalatine artery bifurcation via an external carotid artery stump. Sham-operated animals underwent the same surgical procedure, but the suture was not advanced into the internal carotid artery. In a separate non-survival cohort of animals, laser Doppler flow, arterial blood gases, and mean arterial pressure were monitored to ensure consistency of occlusion and equivalency of physiological variables between groups. Infarction volume was analyzed by 2,3,5-Triphenyltetrazolium chloride (TTC) staining in five 2-mm slices. Infarction volume was determined by SigmaScanPro image analysis system (SPSS Inc) 72 hours after stroke (22.5 hours of reperfusion) as previously described (McCullough et al., 2005).

Assessment of Vascular anatomy

To assess large vessel anatomy in WT, HQ and PARP1^{-/-} mice, three mice of each genotype were deeply anesthetized and perfused via the left ventricle with 5 ml of ice-cold saline followed by 3 ml of FeSO₃ solution (elemental iron: 2 gm/20 ml saline). Mice were decapitated, and brains were removed from the skull with the circle of Willis intact and placed in 10% formalin for 24 hr for examination of the large cerebral vessel anatomy. The development of the posterior communicating artery were quantified on a scale between 0 (no anastomosis) and 3 (fully developed anastomosis) as described previously (Murakami et al., 1998).

Behavioral Scoring

Neurological deficits were scored at 1.5h, 6h, or 48h post-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.

Ovariectomy and hormonal manipulation

In ovariectomized (Ovx) females the ovaries were surgically removed 10 days prior to MCAO. In estrogen treated mice 17 β -estradiol was delivered by subcutaneous SILASTIC capsule (0.062 inch inner diameter; 0.125 inch outer diameter) filled with 0.035 ml of 17 β -estradiol (180 μ g/ml; Sigma) in sesame oil implanted at the time of ovariectomy as described previously (McCullough et al., 2005). Control mice were implanted with oil containing capsules. Serum 17 β -estradiol (E2) was measured by ELISA (IBL HAMBURG, Hamburg, Germany) and uterine weights were recorded at sacrifice to confirm loss of estrogenic effects.

Subcellular fractionation

The SIGMA Nuclei PURE Prep Nuclei Isolation Kit was used for extraction of nuclear proteins. 0.3g brain tissue was placed in 3ml ice-cold Lysis Solution and homogenized using a hand-held Teflon glass homogenizer (40 strokes) on ice. After centrifugation at 800 $\times$ g for 10 minutes, the Pellet (P1) and supernatant (S1) were separately processed further to fractionate nuclei and mitochondria, respectively. For the nuclear fraction, P1 was resuspended in 5.4ml 2.0M sucrose buffer, layered on top of 3.6ml of 2.2M sucrose cushion solution and ultracentrifuged at 30,000 $\times$ g for 45 minutes. The nuclei pellet on the bottom was resuspended in 50 μ l Extraction Buffer (Product Code E 2525, SIGMA-Aldrich, Inc.) for 30 minutes and centrifuged at 20,000g for 5 minutes. The supernatant is the nuclear protein fraction. For the mitochondrial and cytosolic fractions, S1 was centrifuged at 12,000 $\times$ g for 8 min, producing a cytosolic fraction in the supernatant. The pellet was washed with sucrose buffer and centrifuged at 12,000 $\times$ g for 10 min. The final mitochondria fraction was resuspended in sucrose buffer. Each sample represents 4 stroke and 2 sham mice and independent samples were replicated in triplicate.

Western Blotting

Mouse stroke and sham brain samples were obtained by rapid removal of the brain from the skull, resection of the cerebellum, followed by immediate dissection into the right (ischemic) and left (non-ischemic) hemispheres. Each sample represents 4 stroke and 2 sham animals pooled into one sample to allow enough protein for fractionation into nuclear, mitochondrial and cytoplasm protein. Protein concentration was determined using a BCA kit (Bio Rad). 40 μ g of protein was loaded per lane on a 4–15% gradient SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Blots were successively probed, stripped, and reprobed for antigen detection. Antibodies used in this study are anti-AIF antibody (BD Bioscience 57 kDa), anti-Histone antibody (USBiological, Swampscott, Massachusetts, MA), anti-MnSOD antibody (Research Diagnostics Inc, Concord MA), and anti-PAR antibody (1:1000 dilution, from BD Pharmingen). PAR levels were expressed as the ratio to the control actin band with densitometry analysis (Adobe Photoshop 7.0) and all samples were replicated in triplicate (3 independent groups of 4 stroke and 2 sham animals) for statistical analysis. All blots were incubated overnight in primary antibody at 4 °C in TBS buffer containing 5% bovine serum albumin and 0.1% Triton X-100. The secondary antibody was diluted to 1:5,000, and ECL (pico) detection kit (Amersham Biosciences) was used for signal detection.

Statistical Analysis

Data from individual experiments are presented as Mean $\pm$ 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 using SPSSV10 software. $P<0.05$ was considered statistical significant. Induction of ischemia, behavioral and histological assessments were done by a blinded investigator. Due to the phenotype of the Hq mice (hair loss) investigators were genotype and gender blinded after removal of the brain for infarct analysis.

Results

PAR accumulation after stroke

Due to our previous findings of striking sexual dimorphism in PARP-mediated cell death (McCullough et al., 2005), we hypothesized that PARP represents an important branch-point in determining the mechanism by which brain dies after an induced stroke. We began by evaluating PAR polymer formation in wild type (WT) mice after stroke. PAR polymers directly trigger mitochondrial AIF release and lead to direct neurotoxicity *in vitro* (Andrabi et al., 2006; Yu et al., 2006). Nuclear PAR levels began to increase within 2 hours of reperfusion, peaked at 6 hours and began to decline to baseline values by 24 hours (Figure 1a). Levels of PAR were higher in all time points in male mice compared to gonadally intact females, demonstrating the robust activation of PARP in males (Figure 1c). Since estrogen is a known neuroprotectant (Brann et al., 2007), and may directly interact with PARP (Mabley et al., 2005) we assessed the effect of ovariectomy with hormonal control on PAR formation. Ovariectomized females were replaced with either oil or estrogen, and nuclear PAR levels were assessed. As seen in Figure 1b and d, female mice had lower PAR levels compared to age-matched males regardless of hormone status demonstrating that the sex difference in PAR formation was unrelated to estrogen exposure. In order to confirm that the loss of estrogen led to uterine atrophy, we also examined uterine weights in all female mice after stroke, as this parameter may more reliably reflect the physiological response to estrogen exposure. As expected, young ovary-intact female mice and ovariectomized females with estrogen replacement had significantly heavier uteri (91.7 ± 11.5 mg and 100.5 ± 16.4 mg, $n=9$ respectively) than young ovariectomized mice (41.65 ± 11.3 mg, $n=9$, $p<0.05$), as well as significantly higher serum estrogen levels (18.4 ± 3.7 and 24.3 ± 5.8 vs. 4.2 ± 1.5 pg/ml, $p<0.05$).

AIF translocation in WT mice after MCAO

It has been well described by others that stroke induces the translocation of AIF from the mitochondria to the nucleus (Cao et al., 2003; Zhu et al., 2003; Plesnila et al., 2004; Culmsee et al., 2005; Zhu et al., 2007) leading caspase-independent cell death (Susin et al., 2000; Yu et al., 2002). PAR itself is a major trigger for AIF translocation (Andrabi et al., 2006; Yu et al., 2006). As PAR levels are lower in WT females compared to WT males (Fig. 1), the trigger for AIF release may be muted in the female brain, leading to lower levels of AIF translocation and cell death. We first assessed the time course of AIF translocation in male and ovary-intact female WT mice. As can be seen in Figure 2a–d, AIF rapidly translocates from the mitochondrial to the nuclear fragment after stroke. This effect is notable by 2 hours after stroke but consistent with other reports (Plesnila et al., 2004); the translocation becomes more prominent 6 and 12 hours after ischemia. There was no difference in nuclear AIF levels in males and females either at baseline or after stroke. To ensure that differences in AIF levels were not masked by ovarian hormone availability, we then examined AIF translocation 6 hours after stroke in WT male mice and ovariectomized female mice treated with oil or estrogen. There was no effect of estrogen replacement of AIF translocation (Fig 2c and d) demonstrating that AIF translocation, similar to PAR formation, is estrogen-independent.

Sex Differences in Infarct Volumes in PARP1 deficient Mice

To determine the contribution of PARP1 to AIF translocation in male and female mice, we subjected PARP1^{−/−} mice to MCAO. We have previously demonstrated striking sexual dimorphism in the response to a 2hr reversible MCAO in PARP1 deficient mice, with neuroprotection in males and a paradoxical increase in damage in females (McCullough et al., 2005). As prolonged ischemia can lead to overwhelming necrosis, we confirmed this finding using shorter ischemic durations (both 60 and 90 minute MCAO; 90 min shown) and longer survival times (72 hours) to ensure that these sex differences persisted after the acute event. As expected, a significant decrease in infarct size in male PARP1^{−/−} mice was evident compared to WT male mice ($p < .01$) examined 72 hours after injury. However, there was a significant increase in infarct size in female PARP1^{−/−} mice as compared with WT female mice (Figure 3a), implying that the PARP1 pathway is neuroprotective in females.

PAR levels are reduced in PARP1 deficient mice

Although it was expected that PAR levels would be decreased in mice lacking the PARP1 gene, this has not been previously demonstrated *in vivo* after stroke. As many other isoforms of PARP exist we examined PAR formation in PARP1^{−/−} mice after MCAO to ensure that PARP-1 was the primary isoform responsible for PAR formation in both sexes. As seen in Figure 3b and c, deletion of PARP1 leads to a complete amelioration of PAR formation 6 hours after MCAO compared to WT mice. This is equally evident in male and ovary-intact female PARP1^{−/−} animals, confirming that PARP1 is the primary producer of PAR polymers after stroke in both sexes. However, this dramatic reduction in PAR formation led to an increase in ischemic damage (Figure 3a) in PARP1^{−/−} females, demonstrating that cell death in the female brain is unrelated to PAR levels.

AIF translocation is reduced in PARP1 deficient mice

As the translocation of AIF is known to be PARP dependent (Culmsee et al., 2005; Wang et al., 2004), we then examined nuclear AIF translocation in PARP1^{−/−} mice of both sexes 6h after MCAO to ensure that AIF translocation is not triggered by an unknown PARP-independent pathway in females. As seen in Figure 4 loss of PARP1 significantly reduced AIF levels in both male and female mice (Band 1, 2 vs. 3, 4) compared to WT after MCAO. However, this reduction in nuclear AIF translocation had no neuroprotective effect in PARP1

–/– females (as seen in Fig. 3a). Therefore loss of PARP1 reduces PAR formation and stroke-induced AIF translocation in both sexes, but this only leads to neuroprotection in males.

AIF deficient mice also demonstrate ischemic sexual dimorphism

To directly assess the effect of reductions in AIF in male and female mice we proceeded to evaluate harlequin (Hq) mice of both sexes. In agreement with previous studies (Culmsee et al., 2005) Hq males had reduced stroke injury 72 hours after MCAO compared to WT males (Total Infarct: WT 46.3 ± 2.8 vs. Hq $30.5 \pm 2.9\%$ $p > .05$). However Hq females had equivalent ischemic damage to female WT mice (Total Infarct: WT 43 ± 3.2 vs. Hq $37 \pm 3.8\%$, Figure 5), demonstrating that directly reducing AIF does not translate into neuroprotection in the female brain. Behavioral deficits on neurological scoring were consistent with the histological results. Male Hq mice had reduced behavioral deficits compared to WT males (Neuro Scores; Hq $1.9 \pm .03$ vs. WT $2.7 \pm .03$, $p < .05$ at 24 hours), whereas no differences were seen in females (Hq mice $2.0 \pm .03$ vs. WT $2.1 \pm .05$, $p = \text{n.s.}$). Unlike PARP1–/– female mice (Figure 3a), no exacerbation of ischemic injury was seen in female Hq mice compared to their WT (Figure 5). Representative coronal TTC stains (24 hour survival) of gonadally-intact mice of both strains are shown in Figure 6. No differences in physiological measures were present between male, female, WT or Hq mice. In female mice (WT vs. Hq), intra-ischemic pH (7.37 ± 0.02 vs. 7.35 ± 0.01), glucose (149 ± 12 vs. 158 ± 15), mean arterial pressure (81 ± 5 vs. 83 ± 8), hemoglobin (12.7 ± 1.8 vs. 11.9 ± 2.1), O_2 (126 ± 12 vs. 131 ± 9) and CO_2 (40.1 ± 4.6 vs. 38 ± 5.1) were equivalent and within physiological range. This was also found in samples assessed 30 minutes post-ischemia and in males (data not shown). CBF as measured by LDF was equivalently reduced in both cohorts ($n = 4/\text{gp}$; i.e., $11.4 \pm 3.9\%$ of baseline in WT vs. $12.2 \pm 3.4\%$ of baseline in Hq females). Gross vascular anatomy and the number of anastomosis did not differ by strain or sex. No degenerative phenotype (i.e., ataxia) was seen in the Harlequin mice (El Ghouzzi et al., 2007), and no mice were excluded.

Nuclear AIF translocation and PAR formation are reduced in Harlequin mice

To confirm that male and female Hq mice had equivalent reductions in activation of the PARP1/AIF pathway after ischemia we examined the levels of PAR (Figure 7a and b) and AIF (Figure 7c and d) 12 hours after stroke in both WT and Hq mice. Stroke induced a robust increase in PAR formation in WT mice which was significantly decreased in Hq mice of both sexes. The male PAR predominate pattern seen in earlier experiments was also present in both WT and Hq mice. Stroke-induced nuclear AIF translocation was almost completely ameliorated in Hq mice of both sexes (Figure 7c and d) compared to WT.

Discussion

The present study represents the initial steps in the investigation of sex differences in cell death pathways after ischemic injury. Stroke is now the third leading cause of death in the U.S. and the most common cause of disability (Rosamond et al., 2007). The economic burden of stroke is increasing, making the prevention and treatment of stroke a critical public health issue as our population ages. Data is emerging from both *in vivo* and *in vitro* studies that suggest that cell death pathways are fundamentally different in the male and female brain. We have previously shown that deletion of PARP1 or PARP inhibition dramatically reduces damage from an induced stroke in males, but exacerbates injury in females (McCullough et al., 2005). Sexual dimorphism in the response to PARP1 activation has also been demonstrated in both neonatal stroke models (Hagberg et al., 2004) and in the response to endothelial injury after LPS induced inflammation (Mabley et al., 2005) suggesting that PARP is an important point in cell death pathway divergence between the sexes. To further investigate the role of PARP1 in the ischemic brain, we examined the downstream pathways that have been shown to mediate PARP's deleterious effects in males: AIF and PAR polymers.

A large body of evidence shows that PAR formation increases during cerebral ischemia and increases in PAR participate in stroke pathogenesis (Chiarugi, 2005). Deletion of PAR glycohydrolase (PARG), the major enzyme that catalyzes the hydrolysis of PAR into free ADP-ribose leads to early embryonic lethality (Koh et al., 2004). Mice with either a partial deletion of PARG or heterozygote for PARG deficiency survive into adulthood, but are more sensitive to ischemic damage presumably due to the accumulation of PAR polymers (Andrabi et al., 2006; Cozzi et al., 2006). In our first set of experiments we directly examined the formation of PAR in male and female WT mice after MCAO. PAR formation was robust after stroke, but was more pronounced in male mice, especially at later time-points after stroke (Figure 1). Interestingly, a recent clinical study found higher levels of PAR in the CSF of male children compared to females after traumatic brain injury, consistent with our experimental findings after stroke (Fink et al., 2008). As estrogen is neuroprotective, gonadally-intact females have smaller strokes than age-matched males (Alkayed et al., 1998, McCullough and Hurn, 2003) possibly leading to less activation of cell death signaling pathways (ie. PAR formation). To avoid this confound, as well as to directly assess the effect of estrogen on PARP1 signaling and more closely model the population at risk (post-menopausal women), we examined hormonally controlled mice. As seen in Figure 1, PAR formation is induced by stroke equivalently in both groups of females (ovx+oil and ovx+estrogen), demonstrating that PAR formation is unrelated to estrogen levels. As PAR is known to be a major trigger for AIF translocation (Andrabi et al., 2006), we hypothesized that the reduction in PAR formation in females would lead to less translocation of AIF. If females simply have less activation of the NO/PARP/PAR/AIF cell death pathway after stroke, then interference with this system with PARP inhibitors or PARP1 gene deletion would not be as efficacious as in males. Surprisingly, when we directly assessed the amount of nuclear AIF translocation in male and female WT mice, no sex differences were seen. This suggests that AIF translocation can be triggered by non-PAR mediated pathways. This also suggests that the degree of ischemic damage in females is unrelated to changes in AIF translocation. Similar to PAR formation, the amount of nuclear AIF translocation was unaffected by ovariectomy or estrogen supplementation.

We then directly manipulated PAR levels to determine the downstream effects on AIF translocation and ischemic outcome by utilizing PARP1 deficient mice. Female PARP1^{-/-} mice had significantly larger strokes at 72 hours after both 60 (data not shown) and 90 minutes of ischemia (Fig 3a) compared to WT littermates, similar to what has been previously reported with longer ischemic insults (2 hrs.) that lead to pronounced necrosis. We shortened the duration of the ischemic insult and assessed longer survival times to ensure that the sex differences PARP1^{-/-} females were still evident in a model with more apoptotic cell death. Males continued to show a significant neuroprotective effect of PARP1 deletion. Importantly, PARP1^{-/-} females serve as a control for the effects of infarct size on cell death pathway activation, as they have similar infarct volumes to WT males, and significantly larger strokes than PARP1^{-/-} males. This allows for the separation of the degree of damage from activation of cell death, as otherwise findings may simply be correlative (larger strokes leading to more PAR formation, AIF translocation etc). Although PARP-1 is the dominant PARP in brain accounting for 85% of PARP activity, it was possible that other isoforms of PARP were up regulated selectively in PARP1^{-/-} females, leading to high levels of stroke-induced PAR formation or AIF translocation in PARP1^{-/-} females (Schreiber et al., 2006). As seen in Figure 3, this is not the case. Both male and female PARP1^{-/-} mice had a complete absence of PAR formation, showing that PARP1 is the isoform primarily responsible for PAR formation after focal stroke. However, this reduction in PAR only led to protection in males.

PAR is recognized as a trigger for AIF release (Andrabi et al., 2006), the primary inducer of caspase-independent cell death. AIF is a 67 kDa flavoprotein normally located in the mitochondrial intermembranous space with homology to bacterial and plant oxidoreductases (Susin et al., 1999). As it was possible that nuclear translocation of AIF could occur

independently of PARP1 and PAR, and triggered by some unknown cell death signal selectively activated in the female brain, we directly examined AIF levels in PARP1^{-/-} and WT mice after MCAO (Figure 4a and b). Robust nuclear translocation of AIF was seen equivalently in WT mice of both sexes (consistent with data in Figure 2). Importantly, both male and female PARP1^{-/-} mice had dramatic reductions in AIF translocation compared to WT mice (Figure 4e and d). However, in females this reduction in AIF had no relationship to the degree of injury as seen by the lack of neuroprotection in female PARP1^{-/-} mice. This demonstrates that reductions in PARP1, PAR formation, and AIF translocation do not benefit females, and in the case of PARP1, may exacerbate injury.

We then examined stroke outcome in Harlequin (Hq) mice to directly reduce AIF levels and confirm that these sex differences are not unique to PARP1^{-/-} mouse strain. Hq mice have reduced expression of AIF to approximately 20% of WT levels secondary to a retroviral insertion into the first intron of the AIF gene, which interestingly, is located on the X chromosome (AIF^{Hq}, Klein et al., 2002). Consistent with the hypothesis that ischemia-induced PARP activation leads to the translocation of AIF, others have demonstrated a reduction in injury in male Hq mice after a 45 minute MCAO (Culmsee et al., 2005). *In vitro*, knock down of AIF with small interfering RNAs or direct microinjection of AIF-neutralizing antibodies protects mixed sex neurons from glutamate toxicity (Wang et al., 2004; Culmsee et al., 2005). In agreement with this previous work, we found that male Hq mice were protected from a 90 minute MCAO compared to WT males. However, this neuroprotective effect was not present in female Hq mice (Figure 5 and 6). Unlike PARP1^{-/-} mice, Hq females did not have an exacerbation in injury. This could be due to other deleterious effects of PARP activation that are independent of AIF translocation (i.e., NAD depletion; Moroni, 2008) in females, or may be secondary to the residual expression of AIF in this strain. If the PARP/AIF pathway is protective in the female brain, then the residual expression of AIF may be beneficial in females. To ensure that Hq mice had the expected reduction in stroke-induced AIF and PAR formation we directly assessed PAR formation (Figure 7a and 7b) and AIF translocation (Fig 7c and 7d) in Hq mice. Hq mice of both sexes had significant reductions in both PAR formation and AIF translocation after stroke compared to WT littermates.

From these experiments and previous work by others (Hagberg et al., 2004, McCullough et al., 2005, Mabley et al., 2005, Zhu et al. 2006) it is evident that female cell death is mediated by pathways other than PARP and AIF. In the immature brain, this may be related to sex differences in amount of AIF translocation after injury as male neurons displayed a more pronounced translocation of AIF, whereas the female neurons displayed stronger activation of caspase 3 after a HI injury (Zhu et al., 2006). Similar sex-specificity can be modeled in cell culture when sex steroids are removed from the media. 1 After a cytotoxic challenge, programmed cell death proceeded predominately via an AIF-dependent pathway in male (XY) neurons, versus a cytochrome *c*-dependent pathway in female (XX) derived neurons (Du et al., 2004). These sex-specific responses also occur after oxygen-glucose deprivation in hippocampal slice cultures as interference with NO/PARP signaling protected male-derived slices but had no effect in female-derived slices (Li et al., 2005). Our work in adult focal stroke models demonstrates that the amount of AIF translocation does not differ after stroke between the sexes, females simply are resistant to this form of cell death as manipulating nuclear AIF levels has no effect on ischemic outcome.

The question remains that if ischemic cell death is not mediated by PARP/AIF signaling in the female brain, what is the pathway that is predominately activated by ischemia? *In vitro* studies have implicated mitochondrial cytochrome C release, a major triggering event for caspase activation. Neurons derived from females released more cytochrome C after cytotoxic insults (Du et al., 2004) and XX neurons showed a more robust response to treatment with the caspase inhibitor z-VAD *versus* XY neurons. Recent work in neonatal animals has confirmed that

females are exquisitely sensitive to caspase-mediated cell death, as caspase inhibition dramatically reduces injury after a hypoxic-ischemic insult (Renolleau et al., 2007). Recent work in our laboratory has shown similar findings in the adult brain, with robust neuroprotection in females after administration of Q-VD-OPh, a novel, cell-permeable broad-spectrum caspase inhibitor, a drug that had no effect in males (Liu et al., 2009). The possibility exists that by removing PARP or decreasing AIF levels flux increases through the caspase pathway, exacerbating injury in females (as they are more sensitive to caspase-mediated cell death), a hypothesis we are currently evaluating.

Our data demonstrate that PARP activation, PAR formation and AIF translocation occur after stroke in both males and females. However, interference with PARP/AIF signaling has no effect on stroke outcome in females, regardless of hormone status. This suggests that the PARP/AIF pathway does not play a causal role in stroke-induced cell death in females. Therefore pharmacological agents developed to reduce PARP activation may be of limited benefit to females. Recent experimental work with Minocycline, a putative PARP inhibitor that is currently in clinical trials for treatment of acute ischemic stroke, emphasizes this point, as only male animals benefit from treatment (unpublished observations). Recognition and further investigation of these of sex differences will play an important role in the translational success of future neuroprotective agents.

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Figure 1. Nuclear Poly ADP-ribose (PAR) polymer accumulation in WT mice of both sexes after 90 minute MCAO

a. Time course of PAR accumulation in WT mice after stroke. PAR starts to increase from 2 hours after reperfusion, then reaches the highest concentration at 6 hours and gradually decreases by 24 hours. Histone was used as a loading control for the nuclear fraction. b. PAR level at 6 hours after stroke between WT male mice and ovariectomized (OVX) female mice with treatment of Estradiol (E2) or oil implants. PAR levels were higher in male mice regardless of hormone treatment.

c. PAR level in male mice were significantly higher than in female mice from 2 hours to 24 hours after stroke. For each group, $n = 8$ (stroke), 4 (sham), repeated in triplicate. Data represents s.e.m. * $p < 0.01$ and ** $p < 0.05$, # $P < n.s.$

d. PAR levels in male mice were significantly higher than in OVX female mice after treatment with either Estradiol or oil pellets. There was no difference between the Estradiol and Oil treated OVX female mice in PAR formation * $p < 0.01$, ** $p < 0.05$, # $p > 0.05$.

Figure 2. Nuclear AIF Translocation in WT Mice

a. Time course of AIF translocation from the mitochondria to the nuclei in WT mice after stroke. The Mitochondria fraction is shown with MnSOD and the nuclear fraction with Histone showing the purity of the subcellular fractions. Evidence of AIF translocation is seen within 2 hours after MCAO.

b. There was no significant difference in AIF translocation between male and female WT mice ($n=8$ for stroke, 4 for sham, $p>.05$).

c AIF translocation from the mitochondria to the nuclear fragment 6 hours after MCAO in WT male mice and ovariectomized (OVX) female mice treated with Estradiol or oil.

d. No significant difference in AIF translocation was seen between the Estradiol and Oil treatment groups in OVX female mice.

Figure 3. Infarct and PAR formation in PARP1 Deficient Mice

- a. Infarct size of cerebral cortex, striatum and total hemisphere in wild type (n=8) and PARP1 deficient mice (n=10) in both male and female after a 90 minute MCAO 72 hours after stroke. There is a significant decrease in infarct size in male PARP1 deficient mice as compared with wild type male mice ($p<.001$). There is a significant increase in infarct size in female PARP1 deficient mice as compared with wild type female mice ($p<.001$).
- b. Both male and female PARP1^{-/-} mice had a lack of stroke induced PAR formation (lane 1 and lane 2) at 6 hours after stroke
- c. PARP1^{-/-} deficient mice had complete loss of PAR synthesis both in stroke and sham control mice as compared with WT mice. (n=8 per stroke and 4 per sham, samples run in

triplicate). Male WT mice had significantly higher PAR formation compared to female WT mice, consistent with Fig 1 (* $p < 0.01$).

Figure 4. AIF translocation in PARP1 deficient mice

a. AIF translocation from the mitochondria to the nuclear fraction 6 hours after stroke in WT and PARP1 deficient mice of both sexes.

b. Significantly less translocation of AIF from mitochondria to the nuclear fraction occurs in PARP deficient mice as compared with WT mice (Lane 1,2 vs. 3,4), $n=8$ /stroke group, 4 per sham, $p<0.05$. However, no sex differences in AIF translocation are seen either in WT or PARP deficient mice (lane 1 vs. 2 and lane 3 vs. 4, $p=n.s.$).

Figure 5. Infarct in AIF-low expressing (Harlequin) mice

Infarct size of cerebral cortex, striatum and total hemisphere in WT and Hq mice after 90 minute MCAO in both sexes 72 hours after stroke. There is a significant decrease in infarct size in male Harlequin mice as compared with wild type male mice ($n=10$ gp, $*p<.01$, $\#p>.05$). There is no significant difference in infarct size in female Hq mice vs. WT female mice.

Figure 6. Infarction (TTC staining) in Hq mice

Representative coronal sections of Hq and WT mice of both sexes 72 hours after MCAO. White color represents infarcted tissue. Cerebral cortical perfusion was continually monitored by Laser-Doppler flowmetry (LDF/Moor Instruments) in wild type and Hq mice. Cortical perfusion was reduced to approximately 90% of baseline after MCAO and recovered to 70%–80% of baseline after reperfusion in all three strains. The temperature of all mice was kept at 37 ± 0.5 °C throughout the surgery and after care.

Figure 7. PAR Formation and AIF translocation in Harlequin Mice

a. PAR level at 12 hours after stroke in WT mice and Hq mice.

b. PAR levels in Hq mice are significantly reduced compared with WT mice. The same sex pattern (males demonstrating enhanced PAR formation) is seen in Hq and WT mice. PAR levels in males are significantly higher than in female mice in both Hq and WT strains 12 hours after stroke.

c. AIF translocation from mitochondria to nuclear fraction at 12 hours after stroke (CVA) in WT mice and Hq mice of both sexes.

d. Mitochondrial AIF level in Harlequin mice is approximately 20% of WT mice level. Almost no AIF translocation can be detected in the nuclear fraction in Hq mice after stroke.