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Biological Sex and Mechanisms of Ischemic Brain Injury

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Abstract

Cerebrovascular disease is a leading cause of death-from-disease and of disability worldwide, affecting some 15 million people. The incidence of stroke or “brain attack” is unlikely to recede for a decade at minimum by most predictions, despite large public health initiatives in stroke prevention. It has been well established that stroke is also one of the most strikingly sex-specific diseases in its epidemiology, and in some cases, in patient outcomes. For example, women sustain lower rates of ischemic stroke relative to men, even beyond their menopausal years. In contrast, outcomes are worse in women in many clinical studies. The biological basis for this sexual dimorphism is a compelling story, and both hormone-dependent and hormone-independent factors are involved, the latter of which is the subject of this brief review. Understanding the molecular and cell-based mechanisms underlying sex differences in ischemic brain injury is an important step toward personalized medicine and effective therapeutic interventions in patients of both sexes.

Keywords

Stroke; Cerebral ischemia; Gender; Sexual dimorphism; Transient receptor potential M2

Biological Sex and Clinical Stroke and Brain injury

Human ischemic stroke is strikingly sex-specific in its epidemiology (for recent review, see [1]) in that stroke rates are higher in men vs. women, regardless of country-of-origin or population ethnicity [2]. This sexually dimorphic epidemiology persists until ages well beyond the menopausal years, suggesting that the presence or absence of reproductive hormones does not fully account for male vs. female disease patterns. For example, in the large Northern Manhattan Stroke Study, stroke rates in women did not equalize to those of men until beyond 75 years of age [3]. In countries with a high burden of stroke, e.g. Japan, there are clear stratified sex differences in age of first stroke onset, types of risk factors, stroke severity and recovery [4]. Gender differences are also evident in outcomes from treatment, for example in carotid endarterectomy vs. stenting. Symptomatic women sustain higher stroke rates when treated with carotid artery angioplasty and stenting [5].

Nevertheless, stroke risk increases with age in both sexes, and many studies show that outcome from an ischemic event is worse in aged women than in their male counterparts (for comprehensive review, see [6]). Accordingly, stroke is increasingly recognized as a major killer of women, despite the early popular concept that stroke is a “male disease”.

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Importantly, sex differences in stroke risk and pathobiology are present in young children prior to puberty. For example, stroke incidence as estimated from a state-wide hospital discharge database emphasize that boys carry higher risk for all stroke types than do girls [7]. Boys also have a higher case fatality rate for ischemic, but not hemorrhagic, stroke than do girls in this same report. Ischemic stroke is more common in boys, regardless of age, trauma history, or stroke sub-type [8].

The biological basis for this sexually dimorphism in ischemic brain injury is only partially understood. However, both hormone-dependent and hormone-independent factors are likely involved, and selected hormone-independent factors are the subject of this review. The role and mechanisms of female gonadal steroid action in cerebral ischemia and other neurodegenerative diseases have been well-studied to date. Endogenous estradiol and progesterone are both vasoprotective and neuroprotective, less clear are the mechanisms of exogenous female reproductive steroids (for recent mega-analyses, [9, 10]. Despite animal and human observational data that demonstrate estrogen's benefit in stroke and cardiovascular disease, clinical trials such as the Women's Health Initiative have failed to show that exogenous estrogens provide protection for women.

Androgens play an age-specific and dose-sensitive role in the male vulnerability to ischemic injury, although relatively little is known at present about mechanism of action. Again, there are curious data controversies in the animal and human data. For example, in experimental animal studies that control pre- and post-ischemic androgen levels, androgens can reduce [11–13] *or* exacerbate ischemic damage [14–16]. Furthermore, androgens are protective in men after clinical stroke, in that subnormal blood levels of testosterone correlate with increased ischemic damage and poor functional recovery [17–20]. In summary, hormone-dependent mechanisms underlying sex differences in brain injury are significant, and it is likely that chromosomal, genetic sex (XX vs XY based) acts on a molecular platform established early in development by sex steroids. Clinical studies focusing on chromosomal differences are ongoing [21, 22].

The idea is now appreciated that sex differences in the brain's response to ischemic stress can be linked to fundamental cell death mechanisms that are not identical in male vs. female cells. Early data from animal models suggest that favorable outcomes from ischemic brain damage occur more frequently in females and led to in vitro studies of sex-stratified astrocyte or neuron cultures (for additional reviews, see [23–26].

Animal Models of Ischemic Stroke – Sex Differences

Animal data clearly show that the male brain must cope with a heightened sensitivity to cerebral ischemia relative to the female. Most studies have been conducted in rodents, and all focus on tissue outcome rather than risk [27–31]. A groundbreaking study of over 2,000 female and male spontaneously hypertensive and genetically stroke-prone rats showed that development of cerebral hemorrhage and vascular lesions is delayed in females by unknown mechanisms [32]. Subsequently, studies of rats and mice of a variety of inbred and outbred strains demonstrate smaller tissue damage in female brain for an equivalent insult from focal or global cerebral ischemia [30, 33, 34]. Furthermore, experimental stroke in rodent strains with genetic health risk factors such as insulin-dependent genetic diabetes [35], non-insulin dependent diabetes [36], and hypertension [28], all demonstrate that males have enhanced sensitivity to ischemic CNS insults.

In vivo demonstrations of sex-specific ischemic cell death molecules

Recent work has moved beyond comparisons of gross tissue outcomes between the sexes and delineates several sexually dimorphic mechanisms of ischemic cell death. Table 1 lists

examples of molecules that act in a sexually-dimorphic way relevant to cerebral ischemia and neuroprotection. Using genetically altered mice species, these molecules have been shown to vary in importance as death signalers or effectors in males vs. females. A caveat is that only a few studies have distinguished if such diverging molecular signaling pathways are different in the sexes due to hormone –independent or dependent means. For example, the soluble epoxide hydrolase (sEH), an enzyme that is important in the metabolism of vasodilator eicosanoids known as epoxyeicosatrienoic acids (EETS), plays a role in regulation of brain blood flow during ischemia. The sEH is sexually dimorphic in its action during experimental stroke, but this appears to be due to its interaction with estrogen, i.e. a hormone-dependent effect [51]. In the presence of estrogen, sEH expression is suppressed, consequently breakdown of vasodilator EETS is low, allowing the female to enjoy preservation of blood flow relative to the male in brain areas downstream from vascular occlusion.

Most sexually dimorphic molecules have been identified through the use of knock-out mice in which the gene of interest has been eliminated in all cells and tissue types, an approach that can cloud understanding of the cell players involved in the brain injury. With that caveat, the finding that a mutant mouse strain's stroke phenotype is elicited solely in one sex immediately raises the question of whether molecular events during brain injury are identical in both sexes. For example, Angiotensin II type 2 (AT2) receptor signaling protects the brain during cerebral ischemia by multiple mechanisms. Male AT2 deficient mice have larger brain damage after induced stroke, accompanied by a compensatory increase in AT1 receptor expression, than do their male WT counterparts. However, AT2 deficient female mice respond differently to experimental stroke, suggesting that AT2 signaling is protective only in the male brain [39].

Another striking example of an enzyme that acts in a sexually dimorphic way during cerebral ischemia is nitric oxide synthase (NOS). The importance of neuronal NOS in cerebral ischemia is well recognized, in part because ischemia or similar stressors leads to overstimulation of this enzyme and consequent excessive production of nitric oxide leading to toxicity and neuronal death. However, this prevailing hypothesis was established and refined in male animals and mixed-sex neuronal cell cultures. Later studies questioned if NO toxicity was important in the female, revealing that genetic loss of neuronal or inducible (NOS type II) NOS confers robust protection from ischemia in the male brain, but has little effect in the female [31, 47]. As discussed subsequently, the finding that NO-peroxynitrate killing is a key mechanism in male, but not female, ischemic neuronal death has opened investigation to downstream molecular events. This biochemical “pathway” is one of the first to be recognized to be male, but not female, dominant.

Sex Differences in In vitro Models of cell death

Cells obtained from embryos and grown in the absence of sex steroids further reveal an important role of innate sex in sensitivity to injury. Utilizing dopaminergic neurons obtained at embryonic day 14 (E14), Lieb et al. reported that female cells were less sensitive to dopamine toxicity than their male cell counterparts [55]. Du and co-workers provided the first evidence that male and female neurons may proceed to cell death via different pathways [45]. Male neurons are more susceptible to glutamate and peroxynitrite (ONOO⁻) injury than females, while female neurons are more sensitive to staurosporine than male cells [45]. Interestingly, the response to oxidants such as hydrogen peroxide (H₂O₂) is sex-independent [45]. This seminal observation has been expanded to *in vitro* models of cerebral ischemia by exposing male and female neurons in culture to oxygen-glucose deprivation (OGD). Several reports indicate that intrinsic sex contributes to ischemic sensitivity, with most reporting greater sensitivity in male neurons compared to female neurons. Hippocampal slices

cultured from female rat pups exhibit less neuronal cell death as compared to slices from males following OGD [46]. Similarly, female primary hippocampal neurons are less sensitive to ischemia than male neurons [56].

Consistent with these initial reports, male cortical neurons have increased sensitivity to OGD compared to sister cultures [57–59]. However, increased sensitivity in male neurons is not uniformly observed, as it was recently reported that female cerebellar granule neurons (CGN) are more sensitive to OGD than male CGNs [60]. Our data in cerebellar Purkinje cells [61], cortical neurons [54] and hippocampal neurons [62] indicate that cell death following OGD is gender neutral. Further research is required to elucidate if sex-specific sensitivity to ischemia is engaged equally in different neuronal populations as well as other cell types within the brain.

Sex-specific sensitivity to ischemia has also been demonstrated in other cell types in the brain. Recent data indicates that brain microvascular endothelial (EC) cells are differentially sensitive to ischemia, with female brain ECs being relatively resistant to OGD compared to male brain endothelial cells [63]. Similarly, data indicates that cell death after OGD is less in female vs. male astrocytes cultured in the absence of sex steroids [64, 65]. The relative protection observed in female astrocytes is mediated by their relatively high expression of the enzyme P450 aromatase compared to male astrocytes, enabling them to produce the protective steroid 17 β -estradiol. The authors conclude that intrinsic sex difference in astrocyte sensitivity to ischemia is mediated by local steroid synthesis. Again, it is important to note that this observation is not uniformly reported, as astrocyte cell death in response to OGD was recently observed to be sex independent [58]. Regardless, the findings by Liu and colleagues illustrate that dissecting sex-specific differences in ischemic sensitivity independent of sex steroids will prove to be a complicated endeavor and the presence of astrocytes must be carefully considered when studying sex-stratified neurons.

Male cell death – PARP and TRPM2

Recent data indicate that sex-specific neuronal cell death mechanisms exist, indicating the possibility of developing unique neuroprotective strategies for male and female brain. Male cell death after cerebral ischemia appears to be mediated predominantly by excessive NO/ROS production and subsequent over-activation of poly(ADP)ribose polymerase (PARP), whereas female cell death involves caspase-dependent apoptosis. This conclusion comes from data obtained from embryonic cell cultures grown in the absence of sex steroids, implicating genetic sex in determining fundamental cell death mechanisms. However, it remains important to consider the influence of steroids in both the development of these sex differences and their contribution in adulthood. The use of embryonic cells does not completely circumvent the early steroid imprinting of brain cells, as rodents receive two major hormone surges early in development, one around embryonic day 14–16 (E14–16) and another shortly after birth [66, 67]. Therefore, it remains an open question whether the initial hormone surge *in utero* contributes to the development of sex-stratified responsiveness of brain cells to injury in combination with genetic sex. Further, future studies must consider the influence of sex steroids in adult animals in the context of underlying sex-specific signaling. For example, we recently reported that removal of adult testicular androgens abolishes the protection observed in male PARP-1 knockout mice and following PARP inhibition [68]. This intriguing observation points out that understanding the role of sex steroids in shaping ischemic sensitivity must be considered in the context of genetic sex-differences in response to injury.

The most well characterized mechanism of ischemic injury demonstrated to be preferentially engaged in the male brain following *in vivo* and *in vitro* models of cerebral ischemia is the

cascade of events leading to over-activation of PARP and consequent cell death. Figure 1 is a simplified schematic of the PARP-induced cell death pathways; following cerebral ischemia, neuronal nitric oxide synthase (nNOS) is activated, resulting in high levels of nitric oxide (NO) and the consequent buildup of the highly damaging oxidant peroxynitrite (ONOO⁻). Oxidative and nitrosative stress results in damage to DNA which stimulates PARP-1 in the nucleus. PARP-1 contributes to DNA repair by catalyzing the formation of long chains of ADP-ribose (PAR) which are linked to various acceptor proteins, including several important repair enzymes such as DNA ligases, DNA polymerases, histones and PARP itself. A large body of literature has demonstrated that over-activation of PARP-1 is a key contributor to post-ischemic neuronal cell death (hypothesized to be due to energy depletion) and apoptotic death (via apoptosis inducing factor; AIF) (For review see [69, 70]).

Each of the participants in this cell death pathway have been observed to be robustly activated specifically in male cells and animals, from XY neuronal sensitivity to ONOO⁻, to male-specific benefit of nNOS and PARP-1 inhibition and knockout. For example, genetic deletion of PARP isoforms, or administration of PARP inhibitors, reduces ischemic damage in focal stroke models in male animals [31, 71–75]. However, such genetic or pharmacological paradigms fail to protect female brain, and in fact, may increase ischemic damage in females [31, 76]. Similarly, recent data demonstrates that neurons cultured from male PARP-1 knockout mice are protected from OGD, while no benefit was observed in female neurons [60]. Many studies have demonstrated that PARP-mediated cell death following ischemia involves formation of PAR which stimulates mitochondrial release of AIF resulting in caspase-independent apoptosis (for review see [77]). However, it remains an open question whether the level of AIF translocation following ischemia underlies the sex-difference observed in PARP-mediated cell death. Sex differences in AIF activation have been demonstrated in neonatal ischemia [40], however no differences were observed in young adult ischemia models [41, 76]. Therefore, alternative mechanisms may exist downstream of PARP activation that mediate sex-specific damage. Interestingly, we recently demonstrated that PAR itself is neurotoxic [78] and there is evidence that PAR accumulation following cerebral ischemia is sexually dimorphic [41]. PAR is known to stimulate mitochondrial release of apoptosis inducing factor (AIF), however, it remains possible that some of the neurotoxicity of PAR is due to its metabolism to ADPr monomers. PAR is broken down to ADPr monomers by the enzyme poly(ADPr) glycohydrolase (PARG) and PARG inhibition has been shown to protect cells against oxidative stress-induced cell death, implicating ADPr as the mediator of PARP-dependent cell death [79, 80].

A possible downstream mediator of PARP-induced cell death is activation of the non-selective cation channel TRPM2. TRPM2 is a calcium-permeable cation channel that is activated by intracellular ADPr and is expressed throughout the brain, primarily in neurons and endothelial cells. TRPM2 channels are sensitive to hydrogen peroxide (H₂O₂) and contribute to oxidative-stress induced neuronal cell death. We recently demonstrated that genetic knockdown, or inhibition, of TRPM2 protects hippocampal [62] and cortical [54] neurons from OGD in a sexually dimorphic manner, protecting only male neurons. The male specificity of TRPM2 engagement following ischemia and its activation by ADPr provide strong reason to hypothesize that TRPM2 channels are activated following PARP activation and contribute to this cell death pathway (Figure 1). Recent reports indicate that TRPM2 channel activation in response to oxidative stress is dependent on poly(ADP)ribose polymerase (PARP) activity, inferred from the ability of PARP inhibitors to prevent hydrogen peroxide-induced TRPM2 channel activation [81, 82]. However, it remains to be determined in the context of cerebral ischemia whether TRPM2 channel activation requires intact PARP signaling. In conclusion, these observations lead to the hypothesis that ischemia engages neuronal TRPM2 channel activity preferentially in the male, revealing a novel

target downstream of PARP activation that may provide a new approach to neuroprotection. We have recently demonstrated that pharmacological or genetic suppression of TRPM2 channels decrease injury in male mice following experimental stroke [54]. The underlying effect of genetic sex and role of androgens in the adult in the context of early life 'male' imprinting remains an important question regarding the mechanism of TRPM2 channel engagement in ischemic injury and may prove important for dissecting other sex-specific cell death pathways.

Conclusions

We have understood for many years that stroke is a sexually dimorphic disease, and much attention has been focused on the role of sex steroids, particularly estrogen as an explanation for female "protection" (for review see [83, 84]. However, the observation that sexual dimorphism exists in post-menopausal women and pre-puberty indicates that other intrinsic, non-steroidal factors may contribute to ischemic outcome. Rapidly emerging data suggest that different cell death pathways may be engaged after ischemia in male and female brain. This insight affords a new opportunity for the development of novel therapeutics in a gender targeted manner and one basis for the development of personalized medicine.

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Fig 1.

Model of male-specific ischemic cell death and the role of androgen/androgen receptor signaling. Abbreviations are: ADPr, ADP-ribose; AIF, Apoptosis-inducing factor; AR, Androgen receptor, NO, Nitric oxide, ONNO⁻, peroxynitrite; PAR, Poly(ADP-ribose) polymer; PARG, Poly(ADP-ribose) glycohydrolase; PARP, Poly(ADP-ribose) polymerase, TRPM2, Transient receptor potential M2 channel

Table 1**Newly Identified Sex-specific Cell Death Signaling or Effector Molecules**

<u>Molecule</u>	<u>Reference</u>
Akt kinase	[37, 38]
Angiotensin II type 2 receptor	[39]
Apoptosis inducing factor (AIF)	[40, 41]
Caspase 3	[42–44]
Glutathione	[45]
Nitric oxide synthase, neuronal	[46, 31]
Nitric oxide synthase, inducible	[47]
Poly-ADP Ribose Polymerase (PARP)	[31, 48]
P450 Aromatase	[49, 50]
Soluble epoxide hydrolase	[51]
Signal transduction and activator of trans cription-3 (STAT3)	[52]
Superoxide dismutase (SOD)	[53]
Transient receptor potential M2 (TRPM2)	[54]