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Review

Sexual dimorphism in cerebral ischemia injury

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ABSTRACT

Stroke is a leading cause of permanent disability and death. A complex series of biochemical and molecular mechanisms (e.g. the release of ROS/NOS, proapoptotic proteins and proinflammatory cytokine; neuronal depolarization, Ca2+ accumulation and so on) impair the neurologic functions of cerebral ischemia and 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 (17β estradiol or E2) is neuroprotective in many types of brain injury and has been the major focus of investigation over the past several decades. However the incidence of stroke in women is lower than in men until decades past menopause, suggesting that factors beyond sex hormone contribute to these epidemiological sex differences. So a new concept is emerging: both sex steroids and biologic sex are important factors in clinical and experimental strokes. In this review, we will address sex steroids and gender differences in influencing the mechanisms and outcomes of brain ischemia stroke. These sex differences need to be identified which could help future translation to human neuroprotection.

Contents

1. Introduction

Stroke affects 15 million people worldwide each year, and is the leading cause of disability in the United States. The epidemiology of ischemic stroke is sexually dimorphic in that the overall incidence of stroke is higher in men vs. women in most nations

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regardless of country-of-origin and ethnic culture (Sudlow and Warlow, 1997). For example the M:F ratio was 1.5:1.0 in the Hisayama study and females suffer higher risk of stroke than males in patients with IGT and/or smoking. However on the contrary, stroke is an even greater killer in women than in men, since over an entire lifetime, about 16% of women but only 8% of men will die of stroke (Bonita, 1992; Bousser, 1999). The underlying mechanisms involved in these sex differences remain unclear but exposure to gonadal hormones, particularly estrogen, has been thought to play a major role (Hurn et al., 2005; McCullough et al., 2005). In experimental stroke studies, female animals suffer less damage from an induced stroke than males, an effect that can be reversed in part by ovariectomy (McCullough et al., 2003). However, despite preclinical and observational evidence of a protective role for estrogen, recent randomized clinical trials such as the Women's Health Initiative (WHI) have failed to translate the beneficial effects of estrogen into a viable therapy for stroke prevention in post-menopausal women, as treatment with estrogen led to an unexpected increase in stroke rates (Anderson et al., 2004).

This sexually dimorphic epidemiology is also present in children (Golomb et al., 2009) and persists until ages well beyond (>20 years) the menopausal years (Giroud et al., 1991; Sacco et al., 1998), suggesting that not all the observed “female protection” is mediated by steroids. In addition sex-specificity can be modeled in cell cultures grown without background sex steroids. Male neurons, for example, are more susceptible than female cells to challenges from pharmacological insults used to simulate brain injury, e.g., glutamate or peroxynitrite (Du et al., 2004). This differential sensitivity may be related to a relative inability of male cells to maintain intracellular glutathione levels after nitrosative stress. In contrast, response to oxidants such as hydrogen peroxide is gender neutral (Du et al., 2004). These observations do not appear to be limited to neurons. Cell death after oxygen–glucose deprivation is less extensive in female astrocytes (Liu et al., 2007) and in hippocampal slices from females (Li et al., 2005). All these findings suggest that sex-specific sensitivity to cerebral ischemia is partly a function of the sex of cells. The differences are so important that they deserve serious consideration in clinical practice in search for proper diagnostic and therapeutic procedures. For example a report pointed out that there was a higher recanalization rate in females than males following the intravenous t-PA treatment (Savitz et al., 2005). The role of gender on the effects of IV tPA may have important therapeutic implications. In this review we summarize the recent studies about the involvement of sexual dimorphism in cerebral ischemia highlighting the contribution of each study to the field (see Table 1).

2. Estradiol and cerebral ischemia

2.1. Neuroprotective effects of estradiol

It has been known for long that female animals have a lower incidence of naturally occurring stroke than do males. When strains of spontaneously hypertensive animals were followed into old age, female rats had significantly lower rates of spontaneous stroke and death. Surgical loss of estrogen by ovariectomy abolished this benefit, with restoration by estrogen replacement (Yamori, 1976). When cerebral ischemia is induced, females sustain less neuronal damage than do age-matched males, despite equivalent insults (Alkayed et al., 1998). The source of protection is related to female ovarian steroids, because loss of estrogen by either surgical intervention (ovariectomy), pharmacological agents (estrogen receptor antagonists) or aging (reproductive senescence) eliminates this female advantage (Alkayed et al., 1998, 2000; Sawada, 2000). Chronic or acute estrogen treatment reduces damage in these injury paradigms in animals of both sexes (Hurn and Macrae, 2000).

The principal mammalian estrogen, 17β-estradiol, has been most widely studied. Preinjury treatment with 17β-estradiol reduces damage after middle cerebral artery (MCA) occlusion in both sexes. Timing and dose are crucial to outcome in females: (1) treatment with physiological doses for <24 h can be ineffective (Dubal et al., 1998; Liao et al., 2001; Rusa, 1999); (2) acute pharmacological doses of estradiol have robust neuroprotection in male animals, but none to females (Rusa, 1999). Furthermore, low physiological doses are effective in reducing ischemic injury in ovariectomized female rodents, but higher doses lack efficacy.

What is more, implanted with pellets providing physiological levels of 17β-estradiol (E2) beginning 2 weeks before and continuing for 1 week after ischemic insult confers behavioral neuroprotection (Gulinello et al., 2006). Performance on memory tasks that depend on the hippocampus and that show deficits after ischemia was improved by chronic exposure to E2 in ovariectomized rats. Long-term E2 prevented the ischemia-induced deficit in visual working memory, maintaining a normal performance in tests with retention intervals of up to 1 h. Long-term E2 treatment also prevented ischemia-induced deficits in spatial memory tests with short (1 and 7 min), but not longer (15 min) retention intervals.

Estradiol treatment has potential deleterious effects in ischemic injury, despite we know little about it. In one study of histological outcome assessed at 7 days post-injury, chronic estradiol implants delivering very low and physiologically relevant plasma levels of the hormone were found to exacerbate neuronal necrotic cell death in the hippocampal CA1 region (Harukuni et al., 2001). This brain region is known to be “selectively vulnerable” to ischemic injury. CA1 neuronal loss was increased in females or in estradiol-treated surgically ovariectomized rats after global cerebral ischemia. One hypothesis is that estradiol might have differential action in CA1 hippocampus. This would be consistent with previous work using higher doses, which showed estradiol-induced seizure-like activity and increases in N-methyl-D aspartate (NMDA)-binding sites in the CA1 region (Foy et al., 1999).

2.2. Estradiol mechanisms in neuroprotection

At present, there are two known estrogen receptor subtypes, α and β (Kuiper et al., 1996; White, 1987). Both ER-α and ER-β are involved in the mechanisms of neuroprotection. For example chronic administration of selective agonists for either ER-α or ER-β in female rats indicated that the activation of either receptor was able to rescue hippocampal CA1 pyramidal neurons following transient global ischemia (Miller et al., 2005). However, only ERα was upregulated in the CA1 by E2 and ischemia. Researchers also showed that ICI 182,780, a competitive antagonist for both ER-α or ER-β, completely blocks long-term E2 neuroprotection when administered in the early post-ischemic period. These findings confirm that neuroprotection afforded by the chronic treatment with E2 likely involves activation of the classical receptors ER-α and ER-β.

Furthermore ERs have been identified in vascular endothelium, smooth muscle and platelets, so that brain blood vessels must be considered as estrogen targets for cytoprotection. For example, chronic estrogen deprivation increases leukocyte adhesion and infiltration through vascular endotheliumin rat cerebral vessels, exacerbating ischemic neural damage (Santizo and Pelligrino, 1996). Another example is that estradiol treatment increases heat shock proteins (HSP25/27, HSP70) and hemeoxygenase-1 (HO-1) in normal and ischemic brain arteries (Foresti et al., 2001). Estradiol induces endothelial proliferation and migration through

Table 1
The recent studies about the involvement of sexual dimorphism in cerebral ischemia.

Sexual dimorphism	The contribution to the study	Reference
Estradiol	Replacement therapy	Alkayed et al., 1998 Anderson et al., 2004 Dubal et al., 1998 Gulinello et al., 2006 Harukuni et al., 2001 Hurn et al., 2000, 2005 Liao et al., 2001 McCullough et al., 2003, 2005 Rusa, 1999 Wassertheil-Smoller et al., 2003 Yamori, 1976
	NMDA	Foy et al., 1999
	ER	Kuiper et al., 1996 Miller et al., 2005 Santizo and Pelligrino, 1996 Soares, 2003 White, 1987
	HSP/HO-1	Foresti et al., 2001
	STAT	Dziennis and Alkayed, 2008
	ERK/MAPK	Bjornstrom and Sjoberg, 2002 Diane et al., 2009 Segars and Driggers, 2002
	Antioxidation	Culmsee et al., 1999 Horsburgh et al., 2002
Androgens	Replacement therapy	Dash et al., 1991 Herson et al., 2009 Hollander et al., 2003 Liu et al., 2009 Jeppesen et al., 1996 Pan et al., 2005 Uchida et al., 2009 Yeap et al., 2009 Yang et al., 2005
	AR	Patchev et al., 2004
	HSP70	Ahlbom et al., 2001 Chisu et al., 2006 DonCarlos et al., 2006 Lee et al., 2001 Rajdev et al., 2000 Zhang et al., 2004
	Akt	Gatson et al., 2006, Gatson and Singh, 2007
	MAPK	Gatson et al., 2006, Gatson and Singh, 2007 Nguyen et al., 2005
	CREB	Fix et al., 2004 Kousteni et al., 2003 Pike et al., 2008 Unni et al., 2004
XX vs. XY	Neonatal populations	Golomb et al., 2009 Johnston and Hagberg, 2007 Zhu et al., 2006
	Aging populations	Giroud et al., 1991 Hart et al., 1999 Sacco et al., 1998 Wang et al., 2003
	Different response to injury	Du et al., 2004 Li et al., 2005 Lieb et al., 1995 Liu et al., 2007 Zhang et al, 2003 Zhu et al., 2006
	PAR-induced cell death	Hagberg et al., 2004 Lampl et al., 2007 Lang and McCullough, 2008 Li and McCullough, 2009 McCullough et al., 2005 Yuan et al., 2009
	Caspases induced cell death	Le et al., 2002 Liu et al., 2009 Renolleau et al., 2007 Yuan et al., 2009
	Novel cell death pathways	Puyal et al., 2004 Shintani and Klionsky, 2004

interaction with the ER, as demonstrated by evidence of impaired angiogenesis after treatment with ER antagonists (Soares, 2003). Although ligand-bound estrogen receptor (ER) functions as a nuclear transcription factor, estrogen also induces rapid non-genomic cytosolic effects, such as changes in protein phosphorylation/78A. Specifically, estradiol has been shown to rapidly activate STAT1, STAT3 and STAT5 in multiple cell types. The role of STAT3 activation by estradiol has recently been linked to estrogen's neuroprotective effects against cerebral ischemia (Dziennis and Alkayed, 2008).

Estrogen as newly recognized effects on a variety of cytoplasmic signaling cascades, including mitogen-activated protein kinase (MAPK), protein kinase C, phosphatidylinositol-3-OH kinase, and steroid receptor coactivator (src) (Segars and Driggers, 2002). Estrogen binds to a membrane-associated receptor and signals second messenger MAPK cascades, leading to phosphorylation of transcription factors. This enhances transcription of genomic DNA (Bjornstrom and Sjoberg, 2002). E2 can activate the ERK/MAPK pathway in vitro and in vivo (Segars and Driggers, 2002), and intracerebroventricular (icv) injection of the MEK inhibitor PD98059 abrogated the neuroprotective action of chronically administered E2 (Diane et al., 2009). Ischemia induced dephosphorylation of ERK1 and CREB in animals treated with placebo, and E2 treatment maintained p-ERK1 and p-CREB levels in post-ischemic CA1.

The neuroprotective mechanisms of estrogen that are relevant to cerebral ischemia and stroke can be viewed as pan-cellular. The antioxidant activity of estrogen is one of the clearest examples. Fe^{2+}-induced levels of reactive oxygen species and neurotoxicity are significantly attenuated by low mM concentrations of 17β-estradiol and 2-hydroxyestradiol in chick embryonic neurons, even in the presence of the ER antagonist tamoxifen (Culmsee et al., 1999). Some of the effects of estrogen could be mediated by ER-independent activation of apolipoprotein E (Horsburgh et al., 2002).

3. Androgens and cerebral ischemia

3.1. Effects of androgens

Consistent but sparse evidence suggests that male sex and androgens impact ischemic outcomes and mechanisms of brain damage (Herson et al., 2009; Liu et al., 2009). It has been assumed that androgens are detrimental to ischemic pathobiology because male sex is a known stroke risk factor and male animals sustain greater histological damage after experimental stroke than females. Low circulating testosterone levels have also been associated with higher stroke incidence and worse outcomes after stroke in men (Dash et al., 1991; Hollander et al., 2003; Jeppesen et al., 1996; Yeap et al., 2009). Importantly, androgen levels dramatically drop following both experimental and clinical strokes (Dash et al., 1991; Uchida et al., 2009). Thus, ischemia-induced androgen loss may be as important as the steady level of androgens prior to the ischemic insult. In bench studies that control androgen levels, the data are conflicting and indicate that androgens can protect or exacerbate ischemic damage.

Stressors that reduce testosterone levels, e.g. anesthesia administered before the onset of cerebrovascular occlusion, improve ischemic outcomes in the male (Yang et al., 2005). In contrast to the assumed deleterious role of androgen, these studies support the hypothesis that androgens can be neuroprotective in cerebral ischemia. Androgens administered after experimental stroke accelerate functional recovery after stroke (Pan et al., 2005). This latter finding is consistent with clinical data showing that low plasma testosterone in men is inversely associated with stroke severity, infarct size and functional recovery (Jeppesen et al., 1996). We have recently shown that maintaining testosterone or DHT plasma levels within the low physiological range throughout an episode of focal cerebral ischemia confer protection to both adult castrates and gonadally intact aged animals with naturally declining androgens (Cheng et al., 2009; Uchida et al., 2009).

3.2. Neuroprotective mechanisms of androgens

Despite the complexities of androgenic dose–response relationships in ischemia, their neuroprotective properties remain of interest. AR expression has been confirmed in neurons throughout the brain (DonCarlos et al., 2006; Patchev et al., 2004), therefore AR-regulated transcription is a potential mechanism underlying androgen neuroprotection. Although definitive characterization of genes that participate in androgen's neuroprotection has not yet been accomplished, antioxidant proteins and stress-induced heat shock protein HSP70 have been implicated (Ahlbom et al., 2001; Chisu et al., 2006; Lee et al., 2001; Rajdev et al., 2000; Zhang et al., 2004). Androgen non-genomic, rapid signaling pathways also have been implicated in androgen neuroprotection in cerebral ischemia. For example, androgen-induced activation of Akt protects cortical astrocytes against oxidative stress (Gatson and Singh, 2007) and activation of mitogen-activated protein kinases (MAPK) protects hippocampal neurons against cell death induced by Aβ neurotoxicity (Nguyen et al., 2005). In astrocytes and glial cell lines, dihydrotestosterone (DHT) protection is associated with the activation of phosphoinositide-3 kinase (PI3K)/Akt as well as MAPK, while cell membrane impermeable DHT–BSA conjugates suppress MAPK and Akt activation and increase cell death (Gatson et al., 2006; Gatson and Singh, 2007). These data suggest that intracellular AR-dependent activation of protective kinase signaling is important to DHT's actions. Lastly, recent studies emphasize that androgens can rapidly but sustainably activate cAMP response element binding protein (CREB) via non-genomic pathways both in neuronal and non-neuronal tissues (Fix et al., 2004; Kousteni et al., 2003; Pike et al., 2008; Unni et al., 2004). Collectively, these results implicate a possible role of androgen activated non-genomic pathways in androgen neuroprotection following cerebral ischemia. Continued investigation of non-genomic signaling may provide important insights into the mechanisms underlying androgen neuroprotection.

4. XX vs. XY differences to cerebral ischemia

4.1. XX vs. XY cells respond differently to injury

The hormone replacement therapy has been ineffective for both primary and secondary stroke prevention, and chronic estrogen use is associated with higher rates of stroke in treated women (Anderson et al., 2004; Wassertheil-Smoller et al., 2003). This suggests that the replacement of ovarian hormones does not replicate the intrinsic female protection seen throughout much of a woman's life span. Even in neonatal populations, when there are minimal differences in hormone levels between the sexes, males appear to have an “ischemia-sensitive” phenotype (Johnston and Hagberg, 2007; Zhu et al., 2006). Not until women are over age 80 years does their incidence of stroke surpass that of men, suggesting that nonhormonal factors play a role in ischemic sensitivity. Lots of clinical results suggest that not all of the protection afforded to females following ischemic insult are hormonally mediated. For example in patients with atrial fibrillation especially >75 years of age, higher risk is reported in females than males for stroke. (Hart et al., 1999; Wang et al., 2003). Investigation into the mechanisms of cell death has led researchers to believe that the

process of cell death through apoptosis is activated differentially in male and female cells.

In vitro data directly support the related concepts that cell death after injury is sexually dimorphic and that some molecular injury and survival mechanisms are sex-specific. The latter concept has been formulated by studies of male vs. female cell cultures grown without background steroids. In early observations, female dopaminergic neurons were shown to tolerate exposure to toxic dopamine concentrations and survive twofold relative to male cells (Lieb et al., 1995). Similarly, female neurons from the cortical plate or ventricular zone have greater longevity in culture than male cells, and differentially express higher levels of phosphorylated kinases such as Akt (Zhang et al, 2003). Sensitivity to glutamate, peroxynitrate (ONOO) and staurosporine in neuronal culture is sex-specific, with male neurons being more susceptible to glutamate and ONOO than females. In contrast, response to oxidants such as hydrogen peroxide (H_2O_2) is independent of cell sex (Du et al., 2004).

Similar sex specificity is present in the astrocytic response to oxygen and glucose deprivation (OGD) and to toxins that stimulate cell death pathways. Female astrocytes are more resistant to OGD as compared to male cells, but sustain greater cell death when inflammatory mediators are combined with OGD compared to OGD alone (Liu et al., 2007).

4.2. Sex-specific ischemic cell death mechanisms

Many works demonstrate striking sex differences in the cell death pathways triggered by an ischemic insult (Du et al., 2004; Hagberg et al., 2004; Li and McCullough, 2009; Liu et al., 2009; McCullough et al., 2005; Renolleau et al., 2007; Yuan et al., 2009). Although cell death occurs after experimental stroke in both sexes, the response to the injury is different, and these patterns are set in early development (Zhu et al., 2006). To date, all clinical trials of putative neuroprotective agents have failed. This suggests that our understanding of cell death in the ischemic brain is far from complete. Sex differences must be mechanistically defined in order to understand better where, and in whom, neuroprotective therapies will be beneficial.

Apoptotic pathology involves at least two signaling cascades, one initiated through intrinsic, mitochondria-mediated mechanisms involving cytochrome C release, apoptosome assembly and caspase cleavage, and hence is known as a “caspase-dependent” pathway. An alternative caspase-independent pathway is triggered by post-ischemic DNA damage. This caspase-independent pathway involves the activation of poly-ADP ribose polymerase (PARP), release of apoptosis-inducing factor (AIF) from the mitochondria and translocation of AIF to the nucleus.

4.2.1. Poly-ADP ribose-induced cell death

The DNA repair enzyme PARP-1 induces apoptotic death via neuronal nitric oxide synthase (nNOS), PAR, and AIF, which plays a key role in regulating cell death in the male brain (Lang and McCullough, 2008). Cell death induced by activation of nNOS and PARP is clearly sexually dimorphic.

Although nNOS deletion and inhibition were neuroprotective in adult male mice, these manipulations exacerbated stroke damage in adult female mice (McCullough et al., 2005). This sexually dimorphic response was unaffected by hormone exposure/replacement. PARP-1 gene deletion protected male pups in a neonatal hypoxic-ischemic injury (HI) model but had no effects on female pups (Hagberg et al., 2004). Adult females sustain greater damage when PARP-1 is absent (either through genetic deletion or with pharmacological inhibition), suggesting that activational effects or aging itself amplify these sex differences. This exacerbation of injury in adult females when PARP is absent appears to be independent of apparent activation of the PARP-1/AIF pathway (Yuan et al., 2009), because manipulating the levels of PARP-1 or AIF did not correlate with infarct size in the female brain (McCullough et al., 2005). The mechanisms by which sex differences in PARP-1 mediated-cell death occur are unknown. The exacerbation of ischemic injury in PARP-1 knockout female mice may be due to an organizational effect caused by loss of inhibition of the SRY gene activation, leading to a more masculinized ischemic sensitivity pattern. The possible clinical relevance of these findings becomes clear when one considers that minocycline, a putative PARP-1 inhibitor, is currently in clinical trial for treatment of stroke (Lampl et al., 2007). Subsequent work demonstrated that minocycline did not protect ovariectomized female mice despite a robust reduction in infarct in males (Li and McCullough, 2009). Interestingly, PAR polymer formation was reduced equivalently in males and females given minocycline. This again suggests that the relationship between PAR and infarct is nonlinear, at least in females. The relevance of this to clinical populations should serve as a cautionary note for those investigators designing pharmaceutical trials.

4.2.2. Alternative paths to cell death: caspases

Unlike the dramatic neuroprotection seen in males with inhibition of the NO-PARP-1-AIF cell death pathway, females had an exacerbation of injury (Yuan et al., 2009). If females are insensitive to the cell death induced by PARP-1 and AIF, then what triggers apoptosis in the female brain? New evidence suggests that caspases may mediate injury in the female brain after an ischemic insult, although this pathway clearly can occur in both sexes (Le et al., 2002). The best evidence for females' intrinsic sensitivity to caspase-mediated cell death comes from studies utilizing the selective pan-caspase inhibitor QVD-OPH, which is neuroprotective in both neonatal and adult female mice after ischemic injury but has no effect in males (Liu et al., 2009; Renolleau et al., 2007). The neuroprotective effect of QVD-OPH in females is independent of estrogen (Liu et al., 2009).

4.2.3. Novel cell death pathways also show sexual dimorphism

A novel form of cell death that has recently come under study by stroke researchers, autophagy, also appears to exhibit sex differences. Autophagy involves sequestering pieces of the cell's cytoplasm into an autophagosome destined for degradation by the lysosome (Shintani and Klionsky, 2004). Autophagic cell death occurs after neonatal HI in males, as shown by increases in autophagosome formation and lysosome activity, mainly in the penumbra. Autophagic cell death inhibitors are neuroprotective in this model, decreasing ischemic damage (Puyal et al., 2004). Intriguingly, in sex-specific neuronal cultures challenged with nutrient deprivation, male derived neurons readily undergo autophagy and die, whereas female-derived neurons mobilized fatty acids, accumulated triglycerides, formed lipid droplets, and survived longer. Importantly, autophagic inhibitors preferentially protected male-derived neuron.

5. Conclusion

In conclusion, accumulating evidence strongly suggests that biological sex and sex steroids shape both the outcomes of cerebral ischemia and attendant cell death mechanisms. However, many researchers are unaware of the potential confounding effects of sex differences, and much of the preclinical work in stroke continues to focus on young male animals despite the Stroke Therapy Academy Industry Roundtable (STAIR) recommendations that neuroprotective studies be performed in both male and

female rodents (Fisher et al., 2009). Advancing our knowledge of the mechanisms of ischemic cell death and neuroprotective therapies is an important goal in both sexes in order to design and refine our therapeutic targets in a manner beneficial to both sexes, and it is likely that sex-specific therapies are on the horizon.

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