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Research report

HO1 and Wnt expression is independently regulated in female mice brains following permanent ischemic brain injury

Jatin Tulsulkar [a], Alicia Ward [a], Zahoor A. Shah [a,b,*]

[a] Department of Medicinal and Biological Chemistry, University of Toledo, 3000 Arlington Avenue, Toledo, OH 43614, United States
[b] Department of Pharmacology and Experimental Therapeutics, University of Toledo, 3000 Arlington Avenue, Toledo, OH 43614, United States

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ABSTRACT

A gender difference in stroke is observed throughout epidemiologic studies, pathophysiology, treatment and outcomes. We investigated the neuroprotective role of hemeoxygenase (HO) enzyme, which catabolizes free heme to bilirubin, carbon monoxide and biliverdin in the female brain after permanent ischemia. We have previously reported in male mice that genetic deletion of HO1 exacerbates the brain damage after permanent ischemia, and the mechanism of neuroprotection is dependent on the HO1/Wnt pathway; however, the role of HO1/Wnt mediated neuroprotection in the female brain is yet to be investigated. We subjected ovary intact female mice, $HO1^{-/-}$ intact, HO1 inhibitor tin mesoporphyrin (SnMP) treated intact and/or ovariectomized female mice to permanent ischemia (pMCAO), and the animals were sacrificed after 7 days. The SnMP treatment for 7 days significantly reduced the HO1 enzyme activity as compared to that of vehicle treated group. Infarct volume analysis showed significantly lower infarct in intact, $HO1^{-/-}$ intact, and SnMP treated group as compared to the OVX group, suggesting the role of estrogen in neuroprotection. However, there were no differences in infarct volume observed between the intact, $HO1^{-/-}$ and SnMP treated group, suggesting a sexually dimorphic role of HO1 neuroprotection. Western blot analysis on intact and SnMP-treated groups subjected to pMCAO suggested no significant differences in Wnt expression. Together, these results suggest that HO1 neuroprotection is sexually dimorphic and Wnt expression is independently regulated in the female brain following permanent ischemia.

1. Introduction

Stroke is the fifth leading causes of death and permanent disability across the United States. The incidence of stroke is highly dependent on age and sex, and pre- menopausal woman experience a lower stroke risk as compared to men (Sudlow and Warlow, 1997), but after hitting menopause, the incidence and severity of stroke increase in women and become predominant between the ages of 55 and 75 (Mozaffarian et al., 2015). Therefore, it is the aging female population that bears the brunt of reduced stroke-related recovery and institutionalization (Lai et al., 2005). Epidemiological studies suggest that the ovarian hormone, estrogen, is responsible for early neuroprotection in females (Amantea et al., 2005). However, the comparative study of male versus female tissue injury and neurologic deficits at the time of stroke is understudied. A decade of research has shown that stroke is a sexually dimorphic disease, and worldwide databases have consistently shown significantly lower incidence of stroke in females (Gibson, 2013; Manwani and McCullough, 2011) than in males. Stroke kills 16% of post-menopausal females as compared to 8% of males (Mozaffarian et al., 2015). The difference in stroke outcomes between males and females has been found to be largely dependent on reproductive hormones (McCullough et al., 2016).

Experimental stroke studies in rodents have highlighted the loss of neuroprotection in females after removal of the ovaries which was restored upon exogenous estrogen treatment, evidence that estrogen is important for neuroprotection (Dubal et al., 1998). In female animals, tolerance against ischemic brain injury by estrogen is attributed to maintaining and restoring cerebral blood flow, having anti-inflammatory effects and protecting cells from apoptosis (Alkayed et al., 2001; Park et al., 2006; Yang et al., 2005). Experimental data on rodents pertaining to stroke gender differences suggests that cell death after the brain injury may follow different routes depending on sex steroid exposure. The evidence of sexual dimorphism has also been reported by studying inducible nitric oxide synthase (iNOS) expression in male and female mice brains.

* Corresponding author at: Department of Medicinal and Biological Chemistry, University of Toledo, 3000 Arlington Avenue, Toledo, OH 43614, United States
E-mail address: zahoor.shah@utoledo.edu (Z.A. Shah).

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The reduction in ischemic injury was observed in iNOS-null male mice but not in iNOS-null female mice (Park et al., 2006).

Heme oxygenase (HO), also known as heat shock protein 32, is a microsomal enzyme that consists of three different isoforms: 1) inducible hemeoxygenase 1 (HO1), 2) constitutive HO2 and 3) HO3 (Li et al., 2014). The function of HO1 is controlled by number of stimuli, such as the presence of heme, heavy metals, hormones, oxidative stress (Platt and Nath, 1998) and traumatic brain injury (Okubo et al., 2013). The first report on the presence of HO enzymes in liver microsomes was provided by Tenhunen and co-workers (Tenhunen et al., 1968). HO catalyzes the first and rate-limiting step in the oxidative degradation of heme (Fe-protoporphyrin-IX) to carbon monoxide (CO), ferrous ion (Fe^{+2}), and biliverdin-IX (Stocker and Perrella, 2006). HO is not a heme protein but uses heme as both its active center and substrate. CO activates cGMP to induce vasodilation and also acts as a potent anti-inflammatory mediator, whereas biliverdin is converted to bilirubin by bilirubin reductase (Li et al., 2014; Platt and Nath, 1998), also as an antioxidant, and contributes to the protective role of HO1 in ischemia (Sharp et al., 2013). Apart from its role in heme catabolism, HO1 plays important role in various pathological states associated with cellular stress and possesses antioxidant, anti-atherogenic and neuroprotective properties (Platt and Nath, 1998).

In the mammalian genome, the Wnt family is characterized by highly conserved cysteine-rich glycoproteins (Janda et al., 2012). Emerging evidence suggests that Wnt signaling has an essential role in biological processes, including embryonic development and maintenance of stem cells (Yang et al., 2016). Several studies indicate that the cell proliferation, differentiation, migration and central nervous system development is driven by canonical Wnt signaling pathway (Valvezan and Klein, 2012). Moreover, Wnt signaling also plays an important role in the development of neuronal progenitor and stem cells into neurons in male mice (Kuwabara et al., 2009). A recent study showed that HO1 acts upstream of the canonical Wnt signaling cascade (Vanella et al., 2013). In our previous study, we also demonstrated that Wnt expression is modulated by up-regulation of HO1 protein expression, which in turn enhances neurogenesis in male mice.

There were no differences observed in the infarct volume between WT and HO1 knockout ($HO1^{-/-}$) male mice in the transient middle cerebral artery occlusion (tMCAO) model (1 h occlusion, 23 h reperfusion) (Dore et al., 1999). However, studies conducted in our lab (Shah et al., 2011) on male mice showed the delayed ischemic effect (7 days post ischemia) of HO1 in permanent distal middle cerebral artery occlusion (pMCAO) ischemia. We have previously demonstrated that the neuroprotective mechanism of natural product, *Ginkgo biloba* in ovariectomized (OVX) female mice is not dependent on the HO1/Wnt canonical pathway (Tulsulkar et al., 2016). To our knowledge, the role of HO1 in the female brain is yet to be elucidated. Therefore, in this study we hypothesize that the role of HO1 neuroprotection is not beneficial in the female brain after stroke, and that HO1/Wnt pathways are independently regulated in the female brain after pMCAO.

2. Results

2.1. Tin mesoporphyrin (SnMP) administration significantly abrogated HO1 enzyme activity in the female mouse brain

To establish the inhibitory effect of SnMP on HO1 activity, which was determined *ex vivo* by monitoring the conversion of bilirubin in tissue lysates. Brain HO activity was significantly attenuated in SnMP treated female mice (233 ± 57 vs. 55 ± 31 pg/mg/h; $p < 0.05$), demonstrating a reduction of approximately 75% (Fig. 1.). This data suggests that 7 days of continuous administration of SnMP had a significant inhibitory effect on HO activity *in vivo*.

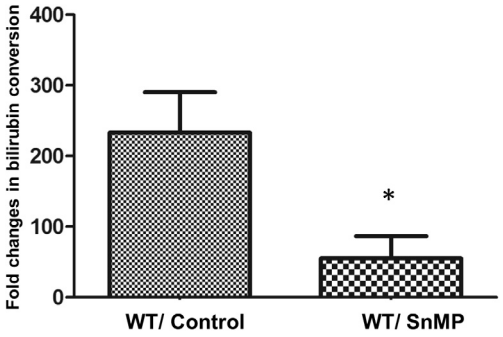

Fig. 1. SnMP treatment attenuates HO activity in female mice brains. Vehicle animals were treated with Na_3PO_4, and the inhibitor group was treated with SnMP (5 mg/kg; i.p) prior to pMCAO and continued for 7 days and sacrificed on day 8. Data are expressed as mean ± SEM; $^{*}p < 0.05$, vs. WT/control n = 3; SnMP intact n = 3.

2.2. Inhibiting HO1 showed no significant differences in brain damage following pMCAO

Our lab has previously reported that $HO1^{-/-}$ male mice had increased infarct volume 7 days after pMCAO (Shah et al., 2011). Nevertheless, the role of HO1 in the female brain after ischemia is yet to be elucidated. Animals were treated with the HO activity inhibitor, SnMP, 24 h prior to pMCAO and continued for 7 days and sacrificed on day 8 (Fig. 2A). $HO1^{-/-}$/Intact, WT/intact and WT/OVX animals also underwent pMCAO and were sacrificed on day 8. Neurological deficits were significantly lower in female animals with intact ovaries ($HO1^{-/-}$ and WT) as compared to those in the WT/OVX group. $HO1^{-/-}$ female animals with intact ovaries did not show any significant differences in neurological deficits as compared to WT/intact mice. To confirm these results, we further used SnMP, an HO activity inhibitor in female WT/intact mice, and then subjected them to pMCAO. SnMP treatment for 7 days did not show significant changes in neurological deficits between WT/intact and intact/$HO1^{-/-}$ females, but the WT/intact, $HO1^{-/-}$/intact and WT/SnMP treated group showed significant reduction in neurological deficits when compared to WT/OVX animals (Fig. 2B). Infarct volume analysis demonstrated that WT/intact, intact/$HO1^{-/-}$ and WT/SnMP group showed no significant differences after 7 days of pMCAO, but all groups were significantly lower than the WT/OVX group (Fig. 2C). Together, these results suggest that HO1 neuroprotection is beneficial only in males and has no role in neuroprotection in the female brains after stroke.

2.3. HO1/Wnt pathway of neuroprotection is independently regulated in female brains after pMCAO

To further understand the effect of HO1 attenuation in the female brains after the 7 days of ischemia, brain tissues were isolated and Wnt protein expression levels were analyzed. The protein expression data shows that SnMP treatment does not affect Wnt expression in female brains after 7 days of pMCAO (Fig. 3A). These results suggest that Wnt expression is independently regulated in the female mice brains.

3. Discussion

In the present study, we demonstrate the dimorphic role of the HO1/Wnt pathway in the female brain after permanent ischemia, which may correspond to gender related differences during stroke. Treatment with the HO1 inhibitor, SnMP, significantly attenuated HO1 enzyme activity. Surprisingly, $HO1^{-/-}$ female mice and SnMP-treated groups showed no significant differences in