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Deletion of macrophage migration inhibitory factor worsens stroke outcome in female mice

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ABSTRACT

Sex is an important factor in the response to ischemic insults in both the laboratory and the clinic. Inflammation and cell death are points where sex-specific pathways diverge in stroke, and serum estrogen level status affect the response to inflammation. The cytokine macrophage migration inhibitory factor (MIF) is detrimental in experimental stroke models in male animals. However MIF is known to have sex-specific actions on inflammation and wound healing. The role of MIF in the ischemic female brain has not been evaluated. A transient middle cerebral artery occlusion (MCAO/90 min) model was used to induce stroke in male, intact female, and ovariectomized female wildtype (WT) and MIF knockout (KO) mice. Infarct size was quantified 72 h after stroke. Protein and cytokine levels were assessed post stroke. Female MIF KO mice had significantly larger strokes compared to WT females (mean hemispheric infarct ± SEM: 63% ± 2% versus 29% ± 3%; $n = 8$; $p < 0.05$). Ovariectomized female MIF KO mice also had larger infarcts than ovariectomized WT littermates (70% ± 3% versus 47% ± 4%; $n = 11$; $p < 0.05$). In males, however, infarct size was equivalent between MIF KO and WT mice (63% ± 2% versus 67% ± 3%; $n = 9$; $p = 0.25$). There were no significant differences in cytokine levels at 6 h post-infarct between mice of either genotype in brain. MIF KO females displayed more microglial activation (ionized calcium binding adaptor molecule 1 (Iba1) immunofluorescence) after stroke than did WT mice or MIF KO males. The larger infarcts in MIF KO females were associated with an early increase in mitochondrial localization of Jun activation domain-binding protein 1 (JAB1). Loss of MIF exacerbated injury in the female brain after experimental stroke, which was independent of changes in pro-inflammatory cytokine levels. This response is sex-specific, and is in part independent of physiological serum levels of estrogen.

Introduction

Each year acute stroke affects approximately 800,000 people in the U.S., with 12% of those patients dying within 1 month of stroke and a third of stroke survivors experiencing permanent disability (Roger et al., 2012). While the incidence of stroke is higher in men than in women throughout most of the lifespan, at older ages more women than men experience strokes (Roger et al., 2012). Estrogen (E2) is a potent neuroprotective agent in vitro and in vivo (Garcia-Segura et al., 2001; McCullough and Hurn, 2003; Stein, 2001). However, randomized clinical trials in postmenopausal women have not shown a benefit from chronic E2 treatment for stroke prevention (Viscoli et al., 2001; Wassertheil-Smoller et al., 2003), in part due to issues with study design (Turtzo and McCullough, 2008). Sex differences in stroke epidemiology and outcome cannot be explained on the basis of circulating levels of gonadal steroids alone (Du et al., 2004; Li et al., 2005; Zhang et al., 2003; Zhu et al., 2006). Laboratory and clinical data indicate that sex (e.g., male versus female) and gonadal steroid exposure both play important roles in the response to an ischemic insult (Turtzo and McCullough, 2008, 2010). Sex differences in ischemic sensitivity may also become more apparent when the influence of gonadal steroids are removed by gonadectomy or with senescence.

Inflammation is a point where sex pathways diverge (Ritzel et al., 2012). A leading candidate for mediating the relationship between inflammation, E2, and sex is the cytokine macrophage migration inhibitory factor (MIF). MIF's effects are mediated via interaction with

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the cell surface receptor CD74 (Savaskan et al., 2012) or through MIF's endocytosis and binding to the intracellular protein JAB1 (also known as COP9 signalsome subunit 5 (CSN5)) (Rendon et al., 2009). MIF promotes inflammation in diseases including sepsis (Bernhagen et al., 1993) and atherosclerosis (Burger-Kentischer et al., 2002; Pan et al., 2004), and mediates the response to oxidative stress (Kudrin and Ray, 2007) and cardiac ischemia (Miller et al., 2008). A clear interaction between MIF and E2 exists in many divergent models including osteoporosis (Hsieh et al., 2007; Onodera et al., 2007a, 2007b; Oshima et al., 2006) and wound healing (Ashcroft et al., 2003).

MIF plays a protective role in acute cardiac ischemic injury, as genetic deletion of MIF increased injury in males in a mouse model of cardiac ischemia (Miller et al., 2008), via inhibition of c-Jun N-terminal kinase (JNK)-mediated apoptosis and a reduction in oxidative cell stress during reperfusion (Luedike et al., 2012). MIF expression is rapidly upregulated in brain after experimental stroke (Wang et al., 2009), and an enriched environment results in the downregulation of MIF in male mice (Inacio et al., 2011c). In contrast to its role in cardiac injury MIF's presence appears to be deleterious in male mice after transient cerebral ischemia, as shown by exacerbation of injury after middle cerebral artery occlusion (MCAO) in male MIF KO mice (Inacio et al., 2011b). As MIF contributes to sex differences in other inflammatory diseases, we hypothesized that genetic deletion of MIF would have different consequences in male and female mice after stroke.

Materials and methods

Animals

The MIF KO mice were from the original MIF genetic deletion (Bozza et al., 1999) backcrossed onto a C57BL6 background (Taylor et al., 2007, 2006). Male and female C57BL6 littermate mice (ages 10 to 12 weeks) were utilized in this study. Genotype was determined by polymerase chain reaction with the following 5′ to 3′ primer sequences, specific for WT and mutant (the knockout allele contains a neomycin marker) alleles, respectively: ACGACATGAACGCTGCCAAC (forward) and ACCGTGGTCTCTTATAAACC (reverse); GAATGAACTG CAGGACGAGG (forward) and GCTCTTCGTCCAGATCATCC (reverse). This study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research. All animal protocols were approved by the Center for Laboratory Animal Care of the University of Connecticut Health Center.

Ovariectomy (Ovx)

Ovaries were surgically removed from female mice 2 weeks prior to middle cerebral artery occlusion (MCAO) and a subcutaneous pellet containing either sesame oil or 17β-estradiol (180 μg/ml) was inserted, yielding physiological levels of E2 (Li et al., 2004; McCullough and Hurn, 2003). Serum E2 levels were measured by ELISA kit (IBL, Hamburg, Germany) and/or uterine weights at sacrifice were recorded to confirm successful ovariectomy.

Middle cerebral artery occlusion (MCAO)

Male and female (intact and ovariectomized (Ovx)) mice (weight 20 to 25 g) were subjected to MCAO as previously described (Sawada et al., 2000). In brief, mice were induced under 4% isoflurane, with maintenance at 1.5% isoflurane. Core body temperature was maintained at 37 ± 0.5 °C. A midline incision was made on the ventral neck, and the right common carotid artery and its bifurcation were exposed. A silicon-coated monofilament suture was introduced through the external carotid artery, passed into the internal carotid artery, and advanced to the root of the right middle cerebral artery (MCA). The animal was allowed to awaken from anesthesia for documentation of neurological deficits to confirm successful occlusion. The animal was then reanesthetized and the suture removed after 90 min of MCAO. Survival (reperfusion) times varied depending upon the cohort examined. Sham surgery was identical to the MCAO protocol except that the monofilament was not inserted into the MCA. After sacrifice, brains were perfused and sectioned, or homogenized for protein extraction. All animals were randomly assigned to sham vs. MCAO cohorts.

Behavioral testing

Neurologic deficit (ND) was scored 30 min after induction of ischemia and again at 6, 24, and/or 72 h. The scoring system was as follows: 0, no deficit; 1, torso turning to the affected side and forelimb weakness when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, either barrel rolling or no spontaneous motor activity (Li et al., 2004).

Physiological assessment

Physiological measurements were performed to compare all cohorts of mice. Monitoring of physiological variables and laser Doppler flowmetry (LDF) was performed in companion cohorts as previously described (n = 4/gp in all genotypes) (McCullough et al., 2004, 2005a, 2005b; Sawada et al., 2000).

TTC staining

Brain slices were incubated in 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C and analyzed by an investigator blinded to mouse sex, hormonal status, and genotype as previously described (McCullough et al., 2004, 2005a, 2005b; Sawada et al., 2000).

Protein lysis/fractionation/Western blot

At 6 h after induction of stroke, brains were rapidly harvested, with removal of the frontal and occipital poles and cerebellum to enrich for territory supplied by the MCA. Brains were frozen at −80 °C until preparation in lysis buffer, and fractionated into cytoplasmic, mitochondrial, and nuclear fractions prior to gel electrophoresis and Western blotting using primary antibodies to MIF (1:2000, Cell Sciences, Canton, MA), JAB1 (1:1000, Abcam, Cambridge, MA), β-actin (1:5000, Millipore, Billerica, MA), histone as a marker of nuclear samples (1:2000, US Biologicals, Swampscott, MA), and cytochrome *c* oxidase (COXIV) as a marker of mitochondrial samples (1:1000, Abcam, Cambridge, MA) as previously described (McCullough et al., 2005b). For fractionation, the infarcted (or sham equivalent) hemispheres of two mice were pooled for each sample.

Co-immunoprecipitation

Co-immunoprecipitation was performed using Protein A-Sepharose beads (generously donated by Elizabeth Eipper, University of Connecticut Health Center). Mitochondrial lysate (100 μg) was incubated with 1 μg of the appropriate antibody (JAB, Abcam or MIF, Cell Sciences) at room temperature for 1 h to form the immune complex. Then 100 μl of a 50% slurry of Protein A beads in PBS was added to the complex and incubated for 1 h at room temperature. Beads were spun down, the unbound fraction removed and then the beads were extensively washed. To elute off the complex, the beads were incubated in sample buffer and boiled. Western blotting was performed to assess the complex as described above.