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Full-length Article

Stress primes microglial polarization after global ischemia: Therapeutic potential of progesterone

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ABSTRACT

Despite the fact that stress is associated with increased risk of stroke and worsened outcome, most preclinical studies have ignored this comorbid factor, especially in the context of testing neuroprotective treatments. Preclinical research suggests that stress primes microglia, resulting in an enhanced reactivity to a subsequent insult and potentially increasing vulnerability to stroke. Ischemia-induced activated microglia can be polarized into a harmful phenotype, M1, which produces pro-inflammatory cytokines, or a protective phenotype, M2, which releases anti-inflammatory cytokines and neurotrophic factors. Selective modulation of microglial polarization by inhibiting M1 or stimulating M2 may be a potential therapeutic strategy for treating cerebral ischemia. Our laboratory and others have shown progesterone to be neuroprotective against ischemic stroke in rodents, but it is not known whether it will be as effective under a comorbid condition of chronic stress. Here we evaluated the neuroprotective effect of progesterone on the inflammatory response in the hippocampus after exposure to stress followed by global ischemia. We focused on the effects of microglial M1/M2 polarization and pro- and anti-inflammatory mediators in stressed ischemic animals. Male Sprague-Dawley rats were exposed to 8 consecutive days of social defeat stress and then subjected to global ischemia or sham surgery. The rats received intraperitoneal injections of progesterone (8 mg/kg) or vehicle at 2 h post-ischemia followed by subcutaneous injections at 6 h and once every 24 h post-injury for 7 days. The animals were killed at 7 and 14 days post-ischemia, and brains were removed and processed to assess outcome measures using histological, immunohistochemical and molecular biology techniques. Pre-ischemic stress (1) exacerbated neuronal loss and neurodegeneration as well as microglial activation in the selectively vulnerable CA1 hippocampal region, (2) dysregulated microglial polarization, leading to upregulation of both M1 and M2 phenotype markers, (3) increased pro-inflammatory cytokine expression, and (4) reduced anti-inflammatory cytokine and neurotrophic factor expression in the ischemic hippocampus. Treatment with progesterone significantly attenuated stress-induced microglia priming by modulating polarized microglia and the inflammatory environment in the hippocampus, the area most vulnerable to ischemic injury. Our findings can be taken to suggest that progesterone holds potential as a candidate for clinical testing in ischemic stroke where high stress may be a contributing factor.

Abbreviations: BDNF, brain-derived neurotrophic factor; F-J C, Fluoro-Jade C; FST, forced swimming test; Iba-1, ionized calcium-binding adapter molecule-1; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MHC-II, major histocompatibility complex II; NFκB, nuclear factor kappa B; NeuN, neuron-specific nuclear antigen; PROG, progesterone; STAIR, Stroke Therapy Academic Industry Roundtable; TBI, traumatic brain injury; TGF, transforming growth factor; TNF, tumor necrosis factor; tPA, tissue plasminogen activator.

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1. Introduction

Stress can trigger, contribute to the development, or worsen the severity of numerous diseases (Slavich, 2016), and epidemiological studies indicate that chronic stress increases risk for stroke incidence and mortality (Everson et al., 2001; Henderson et al., 2013; Everson-Rose et al., 2014; Booth et al., 2015; Sumner et al., 2016). Pre-existing comorbid stress can sensitize/prime the inflammatory response to stroke and thereby worsen outcome and recovery (Stuller et al., 2012; Walker et al., 2014). Animal studies have shown that exposure to stress prior to ischemic stroke exacerbates neuronal damage and inflammation (DeVries et al., 2001; Sugo et al., 2002; Caso et al., 2006, 2007, 2008; Weil et al., 2008; Karelina et al., 2009a,b; Neigh et al., 2009; Norman et al., 2010; Venna et al., 2012).

The inflammatory process in stroke is characterized by rapid activation of resident immune cells, mainly microglia, infiltration of peripheral inflammatory cells, and release of pro-inflammatory mediators into the ischemic brain (Jin et al., 2010). Activated microglia can have either detrimental or beneficial roles depending on their polarization state, or phenotype (Patel et al., 2013; Ma et al., 2016). The M1 phenotype, the classical activation state, exerts neurotoxic inflammatory effects by producing pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α. In contrast, the M2 phenotype releases anti-inflammatory cytokines and neurotrophic factors including transforming growth factor (TGF)-β and brain-derived neurotrophic factor (BDNF), both of which contribute to brain repair (Lee et al., 2014; Jha et al., 2016; Xiong et al., 2016; Dudvarski Stankovic et al., 2016; Zhao et al., 2017). Inhibition of the M1 phenotype and/or stimulation of M2 at strategic time points in the evolution of the stroke have been suggested as potential approaches in the treatment of ischemic injury (Hu et al., 2015; Xia et al., 2015; Amantea and Bagetta, 2016; Dudvarski Stankovic et al., 2016). However, to our knowledge, few translational studies have investigated the efficacy of potential therapeutic strategies targeting the modulation of M1/M2 polarization in ischemic stroke combined with stress.

Long considered "just" a developmental/gestational hormone, progesterone (PROG) is now known to be neuroprotective in numerous experimental models of brain injury, including ischemic stroke (Betz and Coester, 1990; Jiang et al., 1996, 2016; Chen et al., 1999; Murphy et al., 2002; Gibson and Murphy, 2004; Gibson et al., 2005; Morali et al., 2005, 2011; Sayeed et al., 2006, 2007; Ishrat et al., 2009, 2010, 2012; Espinosa-Garcia et al., 2013, 2014; Yousuf et al., 2014a,b, 2016; Remus et al., 2015; Allen et al., 2016; Gaignard et al., 2016; Wali et al., 2014, 2016; Herzog et al., 2017). PROG exerts its neuroprotective effects through multiple mechanisms of action: it stabilizes the blood-brain barrier, reduces brain edema, upregulates GABAergic neurotransmission, decreases excitotoxicity, reduces apoptosis, and downregulates the inflammatory cascade (De Nicola et al., 2013; Guennoun et al., 2015; Siddiqui et al., 2016; Stein, 2017). PROG's anti-inflammatory effects include reduction of pro-inflammatory cytokine expression and regulation of microglia (Gibson et al., 2005; Ishrat et al., 2010; Jiang et al., 2011; Habib et al., 2014; Won et al., 2015; Allen et al., 2016; Lammerding et al., 2016). Our group and others have demonstrated that post-ischemic treatment with PROG attenuates M1 microglial marker expression following hypoxia *in vitro* (Habib et al., 2014) or focal ischemia induced by middle cerebral artery occlusion in rats (Won et al., 2015).

In the current study, we incorporated social defeat stress in our global ischemia model and tested the efficacy of PROG in modulating the effects of comorbid stress on the inflammatory process in the ischemic hippocampus of male rats. We focused on the hippocampus because it is highly sensitive to stress (Conrad, 2008; Kim et al., 2015), and in particular on the hippocampal CA1 region, which is selectively vulnerable to global ischemia (Pulsinelli et al., 1982; Kirino and Sano, 1984; Bartsch et al., 2015; Schmidt-Kastner, 2015). Since prior stress can prime microglia, leading to a potentiated inflammatory response to ischemic stroke, we hypothesized that stress would also dysregulate microglial M1/M2 polarization and worsen the inflammatory environment in the ischemic hippocampus following global ischemia, and that PROG treatment would ameliorate these effects. We evaluated these mechanisms as indicators of PROG's effectiveness in treating ischemic stroke combined with stress.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (250–275 g, Charles River) were individually housed in a climate-controlled room under a 12:12-h reverse light/dark cycle (lights off at 11:00 h), and food and water were provided *ad libitum* throughout the study. Experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol (DAR-2003279) was reviewed and approved by the Institutional Animal Care and Use Committee of Emory University. The experiments are reported here in accordance with the ARRIVE guidelines.

2.2. Experimental design

After a one-week acclimatization period, rats were randomly assigned to one of the following groups: (1) Sham (n = 24), non-stressed controls given vehicle; (2) Sham + PROG (n = 24), non-stressed controls given PROG; (3) Stress + Sham (n = 24), stressed controls given vehicle; (4) Stress + Sham + PROG (n = 24), stressed controls given PROG; (5) ISCH (n = 28), non-stressed animals subjected to global ischemia given vehicle; (6) ISCH + PROG (n = 28), non-stressed animals subjected to global ischemia given PROG; (7) Stress + ISCH (n = 28), stressed animals subjected to global ischemia given vehicle; and (8) Stress + ISCH + PROG (n = 28), stressed animals subjected to global ischemia given PROG. Stressed rats were exposed to 8 consecutive days of social defeat during the light phase of the cycle at the same time of day, while control non-stressed animals remained undisturbed in their home cages. Immediately after last stress session, blood samples were obtained to measure stress hormone corticosterone levels. Depressive-like behavior was evaluated at 24 (pre-test) and 48 h after the end of the stress in the forced swimming test. Next, animals were subjected to global ischemia by the four-vessel occlusion (4-VO) model: cauterization of vertebral arteries (first stage) and transient occlusion of common carotid arteries (second stage); or to sham procedures. Treatment with PROG or vehicle was administered for 7 days after global ischemia or sham surgery. Subgroups of rats in each experimental condition were killed at 7 or 14 days of reperfusion to assess outcome measures using histological, immunohistochemical and molecular biology techniques (see Fig. 1 for graphical representation). All behavioral assessments, biochemical measurements, and quantitative image analyses were performed blind to the experimental group.

2.3. Treatment

Vehicle (20% 2-hydroxypropyl β-cyclodextrin in sterile water; Sigma) or PROG (8 mg/kg/b.wt.; Sigma) were administered by intraperitoneal injection for rapid absorption at 2 h post-

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