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SHORT COMMUNICATION

Estrogen receptor beta signaling alters cellular inflammasomes activity after global cerebral ischemia in reproductively senescence female rats

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Abstract

Periodic treatments with estrogen receptor subtype-β (ER-β) agonist reduce post-ischemic hippocampal injury in ovariectomized rats. However, the underlying mechanism of how ER-β agonists protect the brain remains unknown. Global cerebral ischemia activates the innate immune response, and a key component of the innate immune response is the inflammasome. This study tests the hypothesis that ER-β regulates inflammasome activation in the hippocampus, thus reducing ischemic hippocampal damage in reproductively senescent female rats that received periodic ER-β agonist treatments. First, we determined the effect of hippocampal ER-β silencing on the expression of the inflammasome proteins caspase 1, apoptosis-associated speck-like protein containing a CARD (ASC), and interleukin (IL)-1β. Silencing of ER-β attenuated 17β-estradiol mediated decrease in caspase 1, ASC, and IL-1β. Next, we tested the hypothesis that periodic ER-β agonist treatment reduces inflammasome activation and ischemic damage in reproductively senescent female rats. Periodic ER-β agonist treatments significantly decreased inflammasome activation and increased post-ischemic live neuronal counts by 32% ($p < 0.05$) as compared to the vehicle-treated, reproductively senescent rats. Current findings demonstrated that ER-β activation regulates inflammasome activation and protects the brain from global ischemic damage in reproductively senescent female rats. Further investigation on the role of a periodic ER-β agonist regimen to reduce the innate immune response in the brain could help reduce the incidence and the impact of global cerebral ischemia in post-menopausal women.

Keywords: caspase-1, cerebral ischemia, interleukin 1beta, neuroprotection, NOD-like receptor.

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Estradiol-17β (E_2) is the most potent endogenously synthesized and secreted ovarian estrogen that exhibits anti-inflammatory properties, including the suppression of pro-inflammatory cytokines in the central nervous system (CNS) (Brown *et al.* 2010). In the brain, anti-inflammatory activities of E_2 are mediated by the two classical estrogen receptors (ER), ERα and ERβ (Straub 2007; Suzuki *et al.* 2009; Brown *et al.* 2010). The presence of ER-α or ER-β varies in different tissues, including the brain during aging (Waters *et al.* 2011). For example, in the synapses of the rat hippocampal CA1 region, both ER-α and ER-β decrease with

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Abbreviations used: ASC, apoptosis-associated speck-like protein containing a CARD; C1, caspase 1; DMSO, dimethylsulfoxide; DPN, beta 2, 3-bis(4-hydroxyphenyl) propionitrile; E_2, estradiol-17β; ER-β, estrogen receptor subtype-β; IL-1β, Interleukin-1β; MABP, mean arterial blood pressure; NLR, NOD-like receptor; Ov, ovaries; OvX, ovariectomized; RS, reproductive senescence.

age. In contrast to ER-α, the expression of ER-β increases in response to E_2 in the hippocampus of older animals (Waters *et al.* 2011). The hippocampus is the most vulnerable region in the brain following global cerebral ischemia. A recent study from our laboratory using a rat model of global cerebral ischemia, demonstrated that periodic treatment with an ER-β agonist reduces post-ischemic hippocampal injury in young ovariectomized rats (Raval *et al.* 2013). Since the majority of ischemic events in women occur after the onset of menopause, it is crucial to confirm the efficacy of ER-β agonist treatments in reproductively senescent (RS) females. Furthermore, it has been shown that inflammatory molecules produced during menopause can stimulate innate immune responses in the brain and exacerbate ischemic damage (Brown *et al.* 2010). In young- and middle-aged rodents subjected to ovariectomy, a model that mimics surgical menopause, pro-inflammatory cytokine production increased in several injury models in the both the CNS and the periphery, whereas treatment with physiological levels of E_2 attenuated increases in cytokine production (Vegeto *et al.* 2008; Suzuki *et al.* 2009).

Cerebral ischemia activates the innate immune response, and a key component of the innate immune response is the inflammasome (Abulafia *et al.* 2009; de Rivero Vaccari *et al.* 2014). The inflammasome is a multiprotein complex responsible for the activation of caspase 1 and the processing of the inflammatory cytokines IL-1β and IL-18. The inflammasome is comprised of caspase 1, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and a pattern recognition receptor such as a NOD-like receptor (de Rivero Vaccari *et al.* 2008). In this study, we tested the hypothesis that ER-β regulates inflammasome activation in the hippocampus, and thus reduces global ischemic hippocampal damage in RS female rats that received periodic ER-β agonist treatments.

Material and methods

Animals

All animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the Animal Care and Use Committee of the University of Miami. Animals were purchased from Charles River Laboratories (Wilmington, MA, USA). Adult (6–7 months) or retired breeder (9–12 months) Sprague–Dawley female rats (280–350 g) were purchased, and their estrous cycles were monitored for 14–20 days prior to experimentation by daily examination of vaginal smears (Raval *et al.* 2009). The adult rats were ovariectomized (OvX) using a sterile surgical method and used for the experiment 7 days after ovariectomy (Raval *et al.* 2009). Retired breeder rats that persisted in a single stage for 7 days were considered acyclic. The acyclic rats and rats that remained in constant diestrous were considered RS and were included in this study (Selvamani and Sohrabji 2010). We also confirmed reproductive senescence by virtually undetectable plasma levels of E_2 using a radioimmunoassay kit (Raval *et al.* 2009).

Silencing of hippocampal ER-β after antisense oligodeoxynucleotides (AS) infusion

To knockdown ER-β in the hippocampus, we administered ER-β-AS or scrambled missense [10 nmol of AS mixed with 5 μL of vivo-jetPEI (Polyplus Transfection)] by bilateral cerebroventricular infusion every 24 h for 4 days, as described (Zhang *et al.* 2009; Raval *et al.* 2012). The antisense sequences used in this study were 5′-GAATGTCATAGCTGA-3′ for ER-β or scrambled missense oligo (MS; 5′-ATCGTGGATCGTGAC-3′) used as control. To stimulate estrogen signaling, rats were injected with a single bolus of E_2 (5 μg) on the 2nd day (48 h prior to sacrificing the rats) of AS treatment. Rats were killed on the 4th day of ER-β-AS or MS infusion, and their hippocampal tissue was collected. The paradigm of ER-β-AS infusion and E_2 treatment was adopted from our pervious study demonstrating a significant reduction in ER-β protein levels after treatment with ER-β AS compared with MS control (Raval *et al.* 2012).

Production of global cerebral ischemia

A group of reproductive senescent rats were ovariectomized and exposed to global cerebral ischemia to determine the difference in degree of post-ischemic damage between OvX and RS with intact ovaries (intact Ov) rats. In a subsequent experiment, RS (intact Ov) rats were treated with either an ER-β agonist [beta 2, 3-bis(4-hydroxyphenyl) propionitrile; DPN; 1 mg/kg; s.c. (Waters *et al.* 2009)] or dimethylsulfoxide every 48 h for 10 injections. Forty-eight hours after the last treatment, vehicle- or DPN-treated rats were killed for tissue collection or exposed to global cerebral ischemia.

Global cerebral ischemia was produced by 10 min of bilateral carotid occlusion and systemic hypotension (50 mm Hg) as described (Raval *et al.* 2009). Physiological variables (plasma glucose concentration, pH, PCO_2, PO_2, and mean arterial blood pressure) were maintained at normal levels before and after ischemia. Fourteen days after induction of global cerebral ischemia a histopathological analysis of the brain was performed (Raval *et al.* 2009). Briefly, for each animal, live neurons were counted in the CA1 region of each hippocampus by an investigator blinded to the experimental conditions. Coronal brain sections were made at the level of 3.8 mm from bregma. For each section, 18 fields were obtained and three slides per rat were counted (Raval *et al.* 2009). The data are presented as the mean count from these slides.

Immunoblot analysis

Hippocampi from rats exposed to the various treatment conditions described above were stored at −80°C. At the time of immunoblotting, hippocampi were homogenized; protein content was analyzed and proteins were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described (Raval *et al.* 2009). Proteins were transferred to Immobilon-P (Millipore Corporation, Bedford, MA, USA) membrane and incubated with primary antibodies against ER-β (rabbit polyclonal; 1 : 500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase 1 (mouse monoclonal; 1 : 1000; Novus Biologicals, Littleton, CO, USA) and ASC (mouse monoclonal; 1 : 1000; Santa Cruz Biotech), and IL-1β (1 : 1000, Cell Signaling Technology, Beverly, MA, USA). All data were normalized to β-actin (monoclonal; 1 : 1000; Sigma, St Louis, MO, USA). Immunoblot images were digitized and subjected to densitometric analysis (Raval *et al.* 2009).

Statistical analysis

The data are presented as mean value ± SEM and results from the densitometric analysis were analyzed by a two-tailed Student's *t*-test and a $p < 0.05$ was considered statistically significant.

Results

Silencing of ER-β increased cellular inflammasome proteins

Our previous study demonstrated that the silencing of hippocampal ER-β attenuated E_2 pre-treatment conferred ischemic protection, suggesting that ER-β plays a key role in ischemic neuronal survival (Raval *et al.* 2013). It is known that neuronal death could be triggered by the innate immune response (Adamczak *et al.* 2014). Because cerebral ischemia activates the innate immune response and a key component of the innate immune response is the inflammasome, we hypothesized that the silencing of hippocampal ER-β increases inflammasome proteins in adult ovariectomized rats. Previously, by employing estrogen receptors subtype specific antisense in the hippocampus of female rats, we observed a significant reduction in ER-β protein levels (Raval *et al.* 2012). In this study, we used a similar approach to silence ER-β with an antisense oligonucleotide and stimulated estrogen signaling by E_2. Delivery of E_2 to the ER-β silenced hippocampus of OvX rats resulted in higher inflammasome activation (increased caspase 1 cleavage) and higher expression of the inflammasome adaptor protein ASC when compared with the MS-treated group (Fig. 1a). These data are consistent with higher expression levels of IL-1β, a pro-inflammatory cytokine that is regulated by the inflammasome, in the ER-β-AS-treated group following E_2 treatment (Fig. 1b). These findings indicate that silencing of ER-β attenuates the E_2-mediated decrease in inflammasome activation and pro-IL-1β processing.

Periodic ER-β activation reduce inflammasome activation

Because we observed that the silencing of hippocampal ER-β increased inflammasome proteins, we decided to test if activation of ER-β will reduce cellular inflammasome protein expression in the hippocampus of RS female rats. The hippocampus tissue obtained from RS rats treated with either DPN or vehicle was analyzed for inflammasome protein expression by immunoblotting. Accordingly, we observed a significant decrease in cleaved caspase 1 ($p < 0.002$), ASC ($p < 0.03$), and active IL-1β ($p < 0.02$) in hippocampal lysates of DPN-treated rats (Fig. 1c–f). These data indicate that stimulation of the ER-β reduces inflammasome activation in RS female rats.

Periodic ER-β activation protects hippocampal neurons from ischemic cell death in RS female rats

Because we observed that the periodic ER-β agonist treatments significantly reduced inflammasome proteins in the hippocampus of RS rats, we investigated whether periodic ER-β agonist treatment also reduced post-ischemic hippocampal damage in RS female rats. Before we investigated the effect of periodic ER-β agonist treatments on ischemic outcome in RS rats, we determined if there was any difference in the degree of post-ischemic damage between OvX and RS (intact-Ov) rats. Seven-day OvX and RS

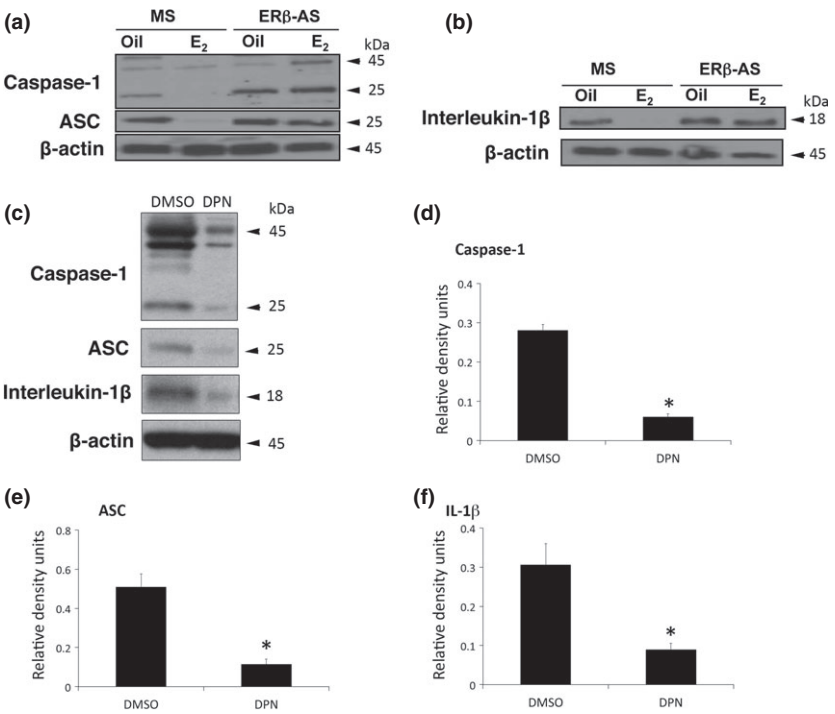

Fig. 1 Estrogen receptors (ER)-β regulates inflammasome activation. Representative immunoblots showing the protein levels of caspase 1 ($n = 6$), apoptosis-associated speck-like protein containing a CARD (ASC) ($n = 6$) (a) and IL-1β ($n = 6$) (b). Lysates were obtained from animals that were treated with either a missense oligonucleotide (MS) or an anti-sense oligonucleotide to ER-β (ER-β-AS). Rats infused with MS or ER-β-AS were injected with a single bolus of E_2 or vehicle-oil to stimulate E_2 signaling. (c) Representative immunoblot corresponding to lysates obtained from animals that were treated either with dimethylsulfoxide (DMSO) or beta 2, 3-bis(4-hydroxyphenyl) propionitrile (DPN) and blotted for caspase 1 ($n = 6$) (d), ASC ($n = 6$) (e) and IL-1 β ($n = 6$) (f). Arrow indicates the active fragments of caspase 1 and IL-1 β (quantified). *$p < 0.05$ as compared to the DMSO treated group.

(intact-Ov) rats were exposed to global cerebral ischemia and histopathological analysis of the brain was performed 14 days later. The results demonstrated no significant difference in the degree of post-ischemic damage between OvX and RS (intact-Ov) rats. Because we did not observe any significant difference in degree of post-ischemic damage between OvX and RS (intact-Ov) rats (Fig. 2), we used RS (intact-Ov) rats to test the effect of periodic ER-β agonist treatments on ischemic outcome. The resulting neuronal count in the CA1 region of the hippocampus revealed that the periodic ER-β agonist treatments significantly increased live neuronal counts to 49% (557 ± 73; $n = 6$) compared to the 17% (190 ± 23; $n = 6$; $p < 0.05$) in the vehicle-treated group (Fig. 2).

Discussion

This study demonstrates that ER-β regulates inflammasome activation and protects CA1 neurons from ischemic damage in RS female rats. The inflammasome is an intracellular multiprotein complex and a key component of the innate immune response. The regulation of the innate immune response is highly complex and involves the activation and regulation of the inflammasome (de Rivero Vaccari *et al.* 2014). Elevation in inflammasome proteins has been previously reported in the hippocampus of aged rats (Mawhinney *et al.* 2011). Consistent with these findings, studies have demonstrated increased pro-inflammatory cytokine levels in middle-aged female rats (Sarvari *et al.* 2014).

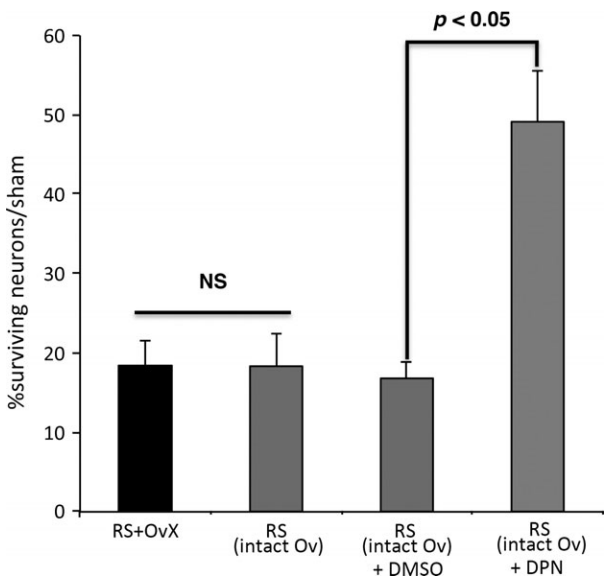

Fig. 2 Beta 2, 3-bis(4-hydroxyphenyl) propionitrile (DPN) increases neuronal survival in the hippocampus after ischemia. Bar graph indicating the number of live neurons in the CA1 region of the hippocampus in reproductively senescent rats that were Ovariectomized (OvX) or rats with intact ovaries that were treated with dimethylsulfoxide (DMSO), DPN or left untreated. Data are expressed as a percentage of sham animals (100% normal neurons) in the rat hippocampus 14 days after induction of global cerebral ischemia.

Inflammasome activation occurs following detection of pro-inflammatory molecules known as damage-associated molecular patterns, or by pathogen-associated molecular patterns. These are sensed by pattern recognition receptors, such as a toll-like receptor or a NOD-like receptor (NLR) (Kigerl *et al.* 2014). The latter, which form inflammasomes such as the NLRP1 (de Rivero Vaccari *et al.* 2008), NLRP2 (Minkiewicz *et al.* 2013), or NLRP3 inflammasome (Halle *et al.* 2008). Once activated, the inflammasome plays an important role in the perpetuation of inflammation in the central nervous system. Previous studies have demonstrated that sex steroids offer neuroprotection through the regulation of the inflammasome (Slowik and Beyer 2015). However, the role of ER-β on the activation of the inflammasome in the CNS remains unknown. In this study we demonstrated that the silencing of hippocampal ER-β increased inflammasome activation; whereas periodic ER-β agonist treatment reduced the activation of the inflammasome, consistent with decreased processing of IL-1β. Importantly, since the knockdown approach used in this study with antisense for ER-β was carried by cerebroventricular infusion; then the silencing of ER-β may not be limited to the hippocampus. Thus, the results we show in this study extended to other parts of the brain as well. Therefore, these findings suggest a role for ER-β on the regulation of the innate immune response through the inflammasome. Future studies will look into which NLRP ER-β interacts with.

In addition to global cerebral ischemia, the inflammasome contributes to the inflammatory response in a variety of conditions and diseases such as Alzheimer's disease (Halle *et al.* 2008) and multiple sclerosis (Soulika *et al.* 2009). These neurological diseases either show sexual dimorphism or an increase in incidence with aging. These are associated with a decline in circulating estrogen in women and with exacerbated central and peripheral inflammation. Therefore, current data showing that ER-β regulates inflammasome activation in the hippocampus of RS female rats suggesting that using a periodic ER-β agonist regimen to reduce the innate immune response in the brain is novel and has broad implications.

Taken together, the role of ER-β agonists on neuroprotection through decreased inflammasome activation has a high therapeutic impact because (i) ER-β remains responsive to E_2 treatment at the CA1 hippocampal synapse after aging, whereas ER-α does not, (ii) ER-β remains active in the brain of RS female rats as observed in this study, and (iii) an ER-β-selective agonist does not stimulate the proliferation of breast or endometrial tissues. Therefore, a periodic regimen of an ER-β agonist treatment could avoid the serious side effects of estrogen replacement therapy in women, such as breast and uterine cancers. Furthermore, sex and age are critical

determinants of ischemic stroke outcomes, and studies assessing sex and age differences as well as the effects of ER-β agonists on inflammasome activation are under investigation.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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