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Estrogen modulates neural–immune interactions through intracellular signaling pathways and antioxidant enzyme activity in the spleen of middle-aged ovariectomized female rats

Prathamesh Kale, Aparna Mohanty, Anushree Patil, Miti Mishra, Uday P. Pratap, Hannah P. Priyanka, Srinivasan ThyagaRajan *

Integrative Medicine Laboratory, Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur 603203, Tamil Nadu, India

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ABSTRACT

Modulation of neural–immune interactions by estrogen in the spleens of ovariectomized (OVX) middle-aged female rats was examined. Con A-induced lymphoproliferation, splenic tyrosine hydroxylase (TH) and nerve growth factor (NGF) expression, levels of p-ERK 1/2, p-CREB, and p-Akt, and activity of superoxide dismutase decreased in OVX rats while estrogen treatment enhanced their expression, levels, and activity. Also, estrogen treatment enhanced Con A-induced IFN-γ production and decreased Con A-induced IL-2 production compared to OVX animals. In contrast, estrogen increased the extent of lipid peroxidation and protein carbonyl formation while OVX induced a decline in protein carbonyl formation. These results suggest that estrogen enhances neural–immune interactions while simultaneously affecting it through generation of free radicals as reflected by increased lipid peroxidation and protein carbonyl formation.

1. Introduction

Development of age-associated diseases such as osteoporosis, cardiovascular diseases, neurodegenerative disorders, and hormone-dependent cancers is common in females due to alterations in gonadal steroids resulting from functional derangement of the neuroendocrine-immune network (Meites, 1990; Koh, 2002; Benz, 2008; McLean, 2009; ThyagaRajan et al., 2012; Vest and Pike, 2013). Normal release of estrogen in young women is altered in middle-aged women before there is a marked decline in its secretion in older women that may result in immunosuppression and subsequent development of diseases (Priyanka et al., 2013a). Estrogen's action to alter the Th1/Th2 immunity toward Th2 type with advancing age may also promote increased incidence of female-specific diseases (Cutolo et al., 2004; Lang, 2004; Salem, 2004). Recently, we have reported that T cell functions were modulated by estrogen in a concentration and its receptor subtype dependent manner through antioxidant enzymes and NO (Priyanka et al., 2013b). In addition, the age-related decline in estrogen secretion associated with cell-mediated immunosuppression may be influenced by deficits in neuronal activity in the hypothalamus and peripheral secondary lymphoid organs (Mohankumar et al., 1994; ThyagaRajan et al., 1995, 2011). The age-related immunosuppression may have been the result of a decline in sympathetic noradrenergic (NA) innervation and norepinephrine (NE) concentration in secondary lymphoid organs of female rats beginning during middle age characterized by irregular estrous cycles (ThyagaRajan et al., 2011).

Another plausible mechanism for the impairment of neuroendocrine-immune system is down-regulation of cellular functions due to damage caused by release of free radicals during the aging process leading to loss of physiological functions. Acceleration in lipid peroxidation with advancing age leads to excessive malondialdehyde formation that binds to cellular membrane proteins affecting their structure and functions that may promote immunosenescence (Esterbauer et al., 1991; Viveros et al., 2007). In addition to an increase in age-associated lipid peroxidation, protein carbonyl compounds accumulate in splenocytes promoting formation of protein cross-links that causes enzyme dysfunctions and disruption of cellular functions in old animals (Tian et al., 1995). Ovariectomy inhibited chemotaxis index, proliferation of lymphocytes, and natural killer cell activity accompanied by increased oxidative stress while estrogen supplementation reversed immunosuppression and cellular oxidative stress in the spleen of rats suggesting the protective effect of estrogen on neuroendocrine-immune functions (Baeza et al., 2009, 2010a, 2010b). Physiological levels of estrogen upregulate antioxidant activity through MAPK and NF-kB pathways to suppress free radical generation and thus, may promote longevity in females (Vina et al., 2011).

Estrogen's diverse actions may be dependent on the dose and duration of the treatment, the organs involved, and also, the role of estrogen

* Corresponding author. Tel.: +91 9940201794.
E-mail address: thyagarajan.s@ktr.srmuniv.ac.in (S. ThyagaRajan).

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receptor subtypes. An increase in number of tyrosine hydroxylase (TH)-positive neurons following estrogen treatment was evident in medial prefrontal cortex of aged female rats and rat proximal urethra while hormone replacement therapy of postmenopausal women and estrogen treatment of ovariectomized female rats reduced the innervation of sympathetic neurons in vagina and uterus, respectively (Zoubina et al., 2001; Smith et al., 2009; Chisholm et al., 2012; Griebling et al., 2012). Estrogen exerts trophic actions to induce neuronal plasticity in hippocampus and improves cognitive performances while its inhibitory effects on innervation in the peripheral organs may be through different class of growth factors and such effects may be determined by the type estrogen and growth factor receptors involved (Hasan et al., 2005; Walf et al., 2011).

Understanding the interaction between estrogen and sympathetic NA neuronal system in the spleen is important for defining the cause of age-related decline in sympathetic NA innervation in secondary lymphoid organs and associated immunosuppression. It is essential to examine the extent of contribution by the compensatory factors such as growth factors and antioxidant enzymes to the impairment of neural–immune interactions in the peripheral lymphoid organs to identify the basis for the development of age-associated diseases in females. Therefore, the present study was conducted to investigate the effects of a 30-day s.c. treatment with placebo or estrogen pellets in OVX middle-aged female rats on cell-mediated immune responses (splenocyte proliferation and cytokine production), neuronal activity (p-TH), growth factor(NGF expression), and associated compensatory mechanisms (antioxidant enzyme activities and NO production) in the spleen.

2. Materials and methods

2.1. Animals

Young and middle-aged female Sprague–Dawley (SD) rats were purchased from the National Institute of Nutrition, Hyderabad and housed for acclimatization at the University's Animal House. The experiments began when the young rats reached the age of 3 months and the middle-aged rats were 8–9 months old. Estrous cycles were monitored by vaginal smears for 8–10 days to establish regular cyclicity in young and middle-aged rats. Food pellets and water were provided *ad libitum* and animals were housed under hygienic conditions. All animal experiments were conducted in accordance with the principles and procedures outlined and approved by the Institutional Animal Ethics Committee.

2.2. Treatment

The middle-aged SD female rats (MA) were either sham operated or ovariectomized and randomly distributed into a sham-operated control group (MA Sham; n = 8), a placebo-treated ovariectomized group (MA OVX + Placebo; n = 8) and two estrogen treatment groups (MA OVX + E 0.6 μg; n = 8 and MA OVX + E 300 μg; n = 8). A separate group of young 3-month old female rats (Young; n = 8) served as age-matched control animals. Initially the middle-aged female rats were either sham operated or bilaterally ovariectomized and after a week of recovery, 30-day placebo or estrogen pellets [17β-estradiol(0.6 μg or 300 μg),Innovative Research America, Florida, USA] were implanted subcutaneously in the nape of the neck. The doses were selected based on preliminary study in our laboratory and a published study (Kasturi et al., 2009). At the end of the treatment period, the animals were sacrificed by decapitation between 08:00 to 10:00 h. The spleen was isolated aseptically and a block was transferred to Falcon tubes containing HBSS (Hanks balanced salt solution) for lymphocyte isolation and other blocks were frozen at −80 °C for biochemical analysis and Western blotting.

2.3. Lymphocyte isolation

Lymphocytes in the spleen were isolated as described previously (ThyagaRajan et al., 1998; Priyanka et al., 2013a, 2013b). A block of spleen was placed in HBSS containing sodium bicarbonate and 4-(2-hydroxyethyl)-1-perazineethanesulfonic acid (HEPES). Spleen tissue was homogenized using a stomacher and the cell suspension was passed through a nylon mesh to remove large aggregates followed by repeated washes with HBSS. Cells were then layered on Histopaque1077 (Sigma-Aldrich, St. Louis, MO) and lymphocytes were removed from the Histopaque/HBSS interface and washed thrice with HBSS. Single cell suspension obtained was counted and 2×10^5 cells/ml were plated in 24 and 96 well-plates for cytokine and proliferation assays.

2.4. Con A-induced lymphocyte proliferation

Lymphocytes isolated from spleen were co-incubated with 0.5 μg/ml, 1.25 μg/ml and 5 μg/ml concanavalin A (Con A) and were kept for 72 h in humidified atmosphere containing 5% CO_2 at 37 °C. Proliferation was assessed using the MTT assay. A 96-well-plate containing 100 μl samples in each well was treated with MTT reagent(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), incubated for 3 h and read at 620 nm after completely solubilizing it in isopropanol containing 37% HCl. Triplicate wells were used for each experimental condition.

2.5. Con A-induced cytokine production

Lymphocytes (2×105 cells/well) co-cultured with or without 1.25 μg/ml of Con A in 24-well plates and kept at 37 °C in an incubator with 5% CO_2 for 24 h. After 24 h, the supernatants and pellets were collected for cytokine assays (IL-2 and IFN-γ) using ELISA kits (R&D Systems, Minneapolis, Minn., USA) and enzyme assays, respectively, and stored at −80 °C.

2.6. Western blot analysis

Western blot analysis has been described previously (Priyanka et al., 2013a). Briefly, Spleen tissue was washed in ice-cold 0.1 M PBS, homogenized in lysis buffer (0.005 M Tris, 0.001 M EDTA,100 μg/ml PMSF, 1 mM activated sodium orthovanadate), centrifuged at 1500 rpm for 15 min and the supernatants obtained were used for Western blotting. Protein concentration was estimated using Folin and Ciocalteu's phenol reagent (Sigma, St. Louis, MO). Sixty micrograms of total protein was electrophoresed on 10% SDS-polyacrylamide gels and blotted on 0.2 μm nitrocellulose membranes (Sigma, St. Louis, MO). The membranes were blocked for 1 h and incubated overnight in blocking buffer containing primary antibody [NGF (M-20; 1:750); p-TH (Ser 40; 1:750) and β-Actin (C4; 1:3000)] (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were then washed with Tris-buffer saline, incubated with HRP-anti rabbit IgG (1:10000) (Santa Cruz Biotechnology, Santa Cruz, CA) and developed using 3,3′,5,5′-tetramethylbenzidine (TMB) Liquid Substrate System (Sigma, St. Louis, MO). Western blotting was performed for at least 7 samples per group and twice for each sample and quantified using densitometry in terms of relative intensity of the blots with reference to control. Signal intensity of the various molecular markers was measured by densitometric analysis using ImageJ 1.45 software (NIH).

2.7. Intracellular signaling pathway markers

Lysis of the spleen tissue was done using 5 mM Tris buffer with phenylmethylsulfonyl fluoride (PMSF) and orthovanadate. After 25 cycles of lysis, the samples were analyzed for ERK 1/2, p-ERK 1/2, CREB, p-CREB, Akt and p-Akt using ELISA (R&D Systems,