Estrogen receptor agonists induce anti-edema effects by altering α and β estrogen receptor gene expression

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The present study aimed to examine whether the attenuation of estrogen receptor expression is prevented by propyl pyrazole triol (PPT), an agonist for estrogen receptor α (ERα) or and diarypropionitrile (DPN), an agonist for estrogen receptor β (ERβ) after traumatic brain injury (TBI). The tests performed on ovariectomized female Wistar rats included sham group, vehicle group, and treated groups: PPT, DPN, and PPT+DPN 30 minutes after TBI. Blood-brain barrier (BBB) disruption and brain water content were estimated. RT-PCR and western blotting were utilized to evaluate ESR1 and ESR2 gene and protein expression. The data indicated that PPT, DPN, and PPT+DPN attenuated TBI-induced brain edema. Also, BBB disruption after TBI was prevented in PPT, DPN, and PPT+DPN-treated TBI animals. Estrogen agonist-treated animals showed a significant elevation in Esr1 mRNA and protein expression levels in the brain tissue of TBI rats. In addition, the data indicated a significant elevation of Esr2 mRNA and protein expression levels in the brain tissue of estrogen agonist-treated TBI rats. The data shows that both ESR1 and ESR2 agonists can enhance ER mRNA and protein levels in TBI animals’ brain. It appears that this effect contributes to the neuroprotective function of ER agonists.

Key words: brain edema, blood brain barrier, ESR1 agonist, ESR2 agonist, traumatic brain injury

INTRODUCTION

One of the most serious brain injuries, even resulting in death and significant disability, is traumatic brain injury (TBI). Several studies have shown significant gender differences in the pathophysiology and occurrence of TBI. The occurrence of cerebrovascular stroke (CS) in males is also higher than in pre-menopausal females (Kim et al., 2019). In older post-menopausal females, the occurrence of CS is the same as in age-matched males (Howe and McCullough, 2015). Some studies have shown that, following TBI, ischemia, and hypoxia, a greater survival rate was observed in young female rodents compared to their male counterparts (Hall and Sutter, 1998; Zhang et al., 1998; Carswell et al., 1999). Neuroprotection has been achieved by estrogen receptor (ER) agonist administration against the background of several experimental neuronal injury models; the treatments decreased the degree of injury and, in a number of cases, reduced behavioral deficiencies and mortality (Asl et al., 2013; Schreihofer and Ma, 2013).

Estrogen, particularly 17 β-estradiol (E2, a potent estrogen), is involved in the development and maintenance of neuronal structure in the central nervous system (CNS) and participates in the regulation of immune system functions such as anti-inflammatory responses in multiple sclerosis disease (Wisdom et al., 2013; Warner and Gustafsson, 2015). Various clinical findings have
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indicated that menopausal women, compared to young women, have a higher vulnerability for brain stroke, cardiovascular disease, and cognition defects, as well as ischemic brain injury and progression of brain edema (Lobo, 1995; Teede, 2003; Kurella et al., 2005). In different neurodegenerative disorders, inflammation is one of the important pathogenic mechanisms involved in disease progression (Fischer and Maier, 2015). Estrogen is an important modulator of the neural inflammatory response in the central and peripheral nervous system (Habib and Beyer, 2015). Several studies have indicated that E2 plays the primary role in the effects of estrogen’s modulation of neuroinflammatory situations, such as TBI (Wise et al., 2001; Khaksari et al., 2011). It has been demonstrated that estrogen can diminish the level of proinflammatory cytokines after TBI, therefore reducing the harmful effects on brain tissue and function (Djebaili et al., 2005; Sarkaki et al., 2013).

Estrogen has different biological functions that are primarily mediated by 2 subgroups of estrogen receptors (ERs); estrogen receptor α (also known as ESR1) and estrogen receptor β (also known as ESR2) (Nilsson et al., 2001). Both ESR1 and ESR2 are expressed in various non-reproductive tissues, e.g., brain, leukocytes, and microglia, indicating a direct pathway for estrogen’s function in inflammatory processes (Sunday et al., 2007; Böttner et al., 2014). In the cortex, E2 regulates neuroinflammatory gene transcription directly through ERs in microglia and astrocytes (Barrett-Connor and Bush, 1991; Sárvári et al., 2011). These ERs are found in regions that could influence neuroprotective effects of estradiol on CNS inflammation after brain edema. Effects of estrogen or ER agonists on brain edema have been confirmed (Naderi et al., 2015). Neuroprotective and anti-inflammatory effects of estrogen, mediated by both ESR1 and ESR2, following TBI in OVX rats have been reported (Khaksari et al., 2015a). It has also been shown that ESR1 plays the main role in Tamoxifen’s neuroprotective effects, in male rats after TBI (Lim et al., 2018). Findings have indicated that ICI 182,780, a non-selective estrogen receptor antagonist, leads to the elimination of estrogen effects on brain blood barrier permeability and brain edema in OVX rats after TBI (Dehghan et al., 2015). In addition, our recent study showed that G-protein-coupled estrogen receptor 1 (GPER, known as GPR30) is also involved in the neuroprotective and cognitive effects of estrogen on the brain (Amirkhosravi et al., 2021).

It has been shown that ESR1 and ESR2 might act to moderate each other (Mazzucco et al., 2006) or act synergistically or antagonistically (Morales et al., 2006; Sinkevicius et al., 2008). Cell populations that express ERs in the CNS have heterogeneity, for example, they are concentrated in neurons, dendritic processes, and astrocytes in the limbic system, cortex, and hippocampus (Su et al., 2001; Milner et al., 2005), but molecular mechanisms of estrogen’s neuroprotective function in the CNS have not yet been fully elucidated.

We have shown previously that propyl pyrazoltriol (PPT), as selective ESR1 agonist, and diarylpropionitrile (DPN), as an ESR2 agonist, have a neuroprotective effect against TBI (Asl et al., 2013). First, the reduction in ESR1 and ESR2 expression after TBI and the inhibitory effect of E2 on this reduction were studied (Khaksari et al., 2015b). Secondly, the exact molecular signaling pathways that cause the neuroprotective function of ER agonists still remain, and the effects of these agonists on ER expression following TBI have also not been reported. Therefore, the current study aimed to investigate whether the attenuation of ER expression is prevented by PPT and DPN after TBI.

This mechanism may reverse the TBI-induced contribution to brain edema and BBB permeability and thus improve estrogen signaling in the inhibition of inflammation after TBI.

METHODS

Animals

In the present study, 35 mature Wistar female rats (weighing 200–250 g) were utilized. The rats were obtained and kept in Kerman University of Medical Sciences’ animal housing. The rats were housed separately in standard polycarbonate cages under controlled lighting (12 h light, 12 h dark cycle) and temperature (23±2°C) conditions, and they had access to standard food and water.

All the test processes were accepted by the Animal Research Ethics Committee of Kerman University of Medical Sciences, Kerman, Iran (Code: K/88/127) in conformity with the internationally adopted principles for laboratory animal care and use, as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) or US guidelines (NIH publication #85–23, revised in 1985).

Bilateral ovariectomy procedure

The female rats were intraperitoneally anesthetized with a ketamine/xylazine mixture (80/10 mg/kg). For ovariectomy, the sub abdominal area was shaved and a cut of 2 cm was made. After opening the skin, fascia, and abdominal muscles, both ovaries were removed. In the end, about 1–2 ml of normal saline solution was shed into the abdomen, then the skin was sutured.
Moreover, before the tests, all the rats were ovariectomized (OVX) for two weeks to prevent the interference caused by the estrus cycle (Khaksari et al., 2013a).

Experimental protocols

The OVX rats were randomly divided into 5 groups before introducing an injury by the TBI technique (7 rats/group), including 1) sham group, animals which were subjected to ovariectomy 2 weeks before the beginning of the test and subjected to false brain trauma under anesthesia but that did not receive any vehicle or hormones. The TBI-OVX groups included 2) vehicle-treated group, two weeks after ovariectomy brain injury was induced and animals were treated with a single dose of DMSO, the ER agonist solvent (0.1 ml; i.p.); 3) PPT-treated group, OVX rats that were treated with a single dose of the ESR1 agonist PPT (2.5 mg/kg; i.p.); 4) DPN-treated group, OVX rats treated with a single dose of the ESR2 agonist DPN (2.5 mg/kg; i.p.); and 5) PPT+DPN-treated group, OVX animals co-treated with a single dose of DPN and PPT (2.5 mg/kg DPN + 2.5 mg/kg PPT in 0.1 ml DMSO; i.p.). The doses of DPN and PPT that were used were selected based on previous investigations that studied female sexual behavior and neurogenesis (Mazzucco et al., 2006; Gonzales et al., 2008; Asl et al., 2013). In groups receiving ESR1 and ESR2 agonists or DMSO, treatment was administered 30 min following brain trauma induction.

Diffuse TBI induction

Before TBI induction all rats were intubated. A diffuse type TBI was induced using an apparatus named TBI induction made by the Department of Physiology, Kerman University of Medical Sciences based on the Marmarou procedure (Khaksari et al., 2013b). In brief, a metal disc (stainless steel, 10 mm in diameter, 3 mm thick) was connected to the skull of an animal that was deeply anesthetized (halothane in 30% O2 gas mixture and 70% N2O), then a 300-g weight was released on the animal’s head from a height of 2 m. A respiratory pump was used for the rats (TSA animal respiratory compact, Germany) immediately following brain injury. After restoring spontaneous breathing, the intratracheal tube was removed and individual cages were used to house the rats following recovery. Diffuse axonal and cellular injury in forebrain structures, such as the hippocampus and sensorimotor cortex, are induced by the TBI model; however, it causes limited damage to the cerebellum and brain stem. The survival rate was approximately 85%.

Brain water content (BWC) determination

Brain water content was used to measure brain edema. Twenty-four hours after TBI induction the rats anesthetized with a ketamine/xylazine mixture (80/10 mg/kg) and then sacrificed and their brain was removed from the skull. The brain samples were weighed before (wet weight) and after (dry weight) 72 h incubation at 60°C. Then, the water content percentage of each brain sample was calculated by the following formula: (100 × [(wet weight - dry weight)/wet weight]) (O’Connor et al., 2005).

Determining blood-brain barrier (BBB) disruption

By evaluating the leakage of Evans blue (EB) dye, the degree of BBB disruption was measured, conforming to the O’Connor protocol (2005). Briefly, PBS 0.01 M with a concentration of 2% was used as EB dye solvent, then 2 ml/kg of dye as a BBB permeability detector was injected into the rat’s tail vein, 4 h after TBI. For removing the intravascular EB dye, rats were re-anesthetized 5 h with halothane and transcardiac perfusion was performed with 200 ml of heparinized saline via the left ventricle. The animals’ brains were also removed and 1 ml of PBS was used to homogenize the samples, then 0.7 ml of 100% (w/v) trichloroacetic acid was added and the samples centrifuged (30 min per 1000 g). Then, the absorption of EB dye in the supernatant was evaluated at 610 nm via a spectrophotometer (UV/VIS, Spectrometer, UK). The measured value of extravasated EB dye was used to indicate µg/g brain tissue.

Dissection and tissue preparation

CO2 exposure was used to anesthetize the animals and then they were decapitated. Their brains were dissected and the tissue samples were weighed, then they were immediately frozen in liquid nitrogen and stored at -70°C until use in the experiment.

mRNA analysis

From the brain tissue, total cellular RNA was separated by a modified guanidine isothiocyanate–phenol-chloroform procedure via RNX+ reagent (Sakhaie et al., 2020). The method used for this work was semi-quantitative RT-PCR. Briefly, total RNA (5 µg) was combined with Oligo-dT primer M-MuLV RNA-dependent DNA polymerase was used for the RT-PCR reaction (60 min incubation at 42°C and 10 min inactivation at
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70°C) relying on the manufacturer’s protocol (Fermen‑
tas GMBH, Germany). In order to study gene expression
in the brain tissue, three separate PCR reactions were
used. Selective forward and reverse primer sequences
for Esr1, Esr2, and Actb (β‑actin, as an internal standard)
were used to perform the reaction in each PCR. The
primer sequences were: 

- Esr1 forward: 5’‑TAC AGC AAC ACC ATC CAG TC‑3’,
- Esr1 reverse: 5’‑AAG TGG GTT TCT AGC ATG CC‑3’,
- Esr2 forward: 5’‑AGT TCC AGG ACA AAG TC‑3’,
- Esr2 reverse: 5’‑GGA TGA TGT CAC GGC CAG TC‑3’,
- Actb forward: 5’‑CCC AGA GCA AGA GAG GCA TC‑3’,
- Actb reverse: 5’‑CTC AGG AGG AGC AAT GAT CT‑3’.

Taq DNA polymerase (Roche, Germany) was used
for DNA amplification and the reactions were regulat‑
ed according to the manufacturer’s protocol. The PCR
reactions were also cycled (25 cycles) after incubation
at 94°C for 5 min via the following temperature pro‑
file: (94°C for 45 s, 55°C for 45 s, and 72°C for 45 s). An
extension step at 72°C for 5 min was performed fol‑
lowing the last cycle. The PCR products were separat‑
ed via electrophoresis in a 1.5% agarose LMMP (Roche,
Germany) gel, and densitometry was used to quantify
bands through Lab Works analysis software (UVP, UK).

Finally, in the brain tissue samples for estimation of
ER mRNA levels, the semiquantitative PCR method
was used, which was normalized to β‑actin (a standard
housekeeping gene).

Protein analysis

The homogenization of brain sample tissue was per‑
formed in RIPA buffer, including 10 mM Tris–HCl (pH
7.4), 0.1% SDS, 1mM EDTA, 1% NP‑40, 0.1% Na‑deoxy‑
cholate, with protease inhibitors (1 mM phenylmeth‑
ylsulfonyl fluoride, 2.5 µg/ml of leupeptin, 1 mM so‑
dium orthovanadate, and 10 µg/ml of aprotinin). The
homogenized tissue was centrifuged at 14,000 rpm at
4°C for 15 min. The resulting supernatant was pre‑
served as the total‑cell fraction. The Bradford method
was used to evaluate protein concentrations (Bio‑Rad
Laboratories, Munich, Germany). Bovine serum albu‑
min (BSA) was used as the protein standard in the Brad‑
ford assay. Equal protein volumes (40 µg) were loaded
on a 9% SDS‑PAGE gel, then they were transferred to
PVDF membranes. Blocking buffer (20 mM Tris–HCl, pH
7.5, 150 mM NaCl, 0.1% Tween 20) was used to block
the membranes overnight at 4°C with 5% non‑fat dried
milk in Tris‑buffered saline with Tween 20, and then
primary antibodies were used to probe them ESR1 and
ESR2 (1:1000, Santa Cruz, USA) for 3 h at room tem‑
perature. The membranes were washed in TBS‑T buffer
(3 times for 5 min) and incubated with a horseradish
peroxidase‑conjugated secondary antibody for 60 min

at room temperature (1:15,000, GE Healthcare Bio‑Sci‑
ences Corp. NJ, USA). The ECL system was used for the
detection of antibody‑antigen complexes with exposure
to Lumi‑Film chemiluminescent detection film (Roche,
Germany). The expression intensity was analyzed with
Lab Work analysis software (UVP, UK). To control for
loading, β‑actin (1:1000, Cell Signaling Technology, INC.
Beverly, MA, USA) was applied.

Statistical analysis

The results are shown as mean ± SEM. One‑way anal‑
ysis of variance (ANOVA) as well as the Tukey post hoc
test, were used to distinguish the difference in EB leak‑
age, ER expression level between experimental groups,
and brain water content. A p‑value less than 0.05 was
considered statistically significant.

RESULTS

The effects of ER agonists on BBB disruption
and brain edema

As shown in Table 1, the percentage of BWC in the
TBI group that received vehicle was greater than the
sham group (p<0.001). A significant attenuation of in‑
creased water content was observed in the PPT‑treated
(p<0.01), DPN‑treated (p<0.01), and PPT+DPN‑treated
(p<0.001) groups in comparison to the vehicle‑treated
group. Moreover, the amount of this indicator in the
combined group was significantly lower compare to
DPN‑ or PPT‑treated groups (p<0.001).

The brain EB content in the vehicle‑treated group
increased after TBI in comparison to the sham group
(p<0.001). However, this increase in brain EB con‑
tent following TBI was significantly inhibited in
PPT‑treated (p<0.01), DPN‑treated (p<0.01), and PPT+DPN‑treated
(p<0.001) groups in comparison to the vehicle‑treated
group. Moreover, the amount of this indicator in the
combined group was significantly lower compare to
DPN‑ or PPT‑treated groups (p<0.001) (Table 1).

The effects of ER agonist treatment on the
expression of Esr1 mRNA and protein

In PPT‑treated (29%), DPN‑treated (32%), and
PPT+DPN‑treated (40%) groups, Esr1 mRNA levels in‑
creased in brain tissue in comparison to the sham
or vehicle‑treated groups (respectively, p<0.01; p<0.01;
p<0.001) (Fig. 1). The same effect was observed for
ESR1 protein, as this protein in PPT-treated (55%), DPN-treated (61%), and PPT+DPN-treated (94%) groups was increased in comparison to the vehicle-treated animals (p<0.001). The effect of the combined group on the ESR1 protein level was significantly higher compared to the PPT- or DPN-treated rats (p<0.05) (Fig. 2).

The effects of ER agonist treatment on the expression of Esr2 mRNA and protein

As shown in Fig. 3, Esr2 mRNA expression in the brain tissue significantly decreased in the vehicle-treated group in comparison to the sham group (p<0.01). In PPT-treated (94%), DPN-treated (96%), and PPT+DPN-treated (100%) groups, Esr2 mRNA levels were increased in brain tissue compared to the vehicle-treated group (respectively, p<0.01; p<0.01; p<0.001) (Fig. 3). The western blot data also indicated that ESR2 protein expression was significantly reduced in the vehicle-treated versus the sham group (p<0.001). Furthermore, in PPT-treated (82%), DPN-treated (81%), and PPT+DPN-treated (100%) groups, ESR2 protein expression was elevated in brain tissue compared to the vehicle-treated group (respectively, p<0.05; p<0.05; p<0.001). The effect of the combined group on ESR2 protein level was significantly higher compared to the PPT- or DPN-treated rats (p<0.05) (Fig. 4).

**DISCUSSION**

In the current study, roles for ESR1 and 2 expressions were investigated by using PPT and DPN (as ESR1 and ESR2 agonists) in ovariectomized TBI rats. The main findings of this study include: 1) the ESR1 agonist PPT and ESR2 agonist DPN reduced brain edema and BBB disruption after TBI and the effect of the combined group PPT+DPN was higher than administration of ER agonists alone; 2) both estrogen receptor 1 mRNA and protein levels increased following PPT treatment in TBI animals, and estrogen receptor 2 mRNA and protein levels were increased after DPN treatment in TBI animals; and 3) the effect of the combined group on ESR1 and ESR2 protein levels was higher than for the individual use of these agonists.
The present study showed both ER agonists had a neuroprotective effect via decreasing brain edema and suppressing BBB disruption after TBI. Previous reports illustrated that E2 exhibits a healing effect on neurological damage, including brain edema, BBB disruption, and intracranial pressure (ICP) after TBI (Asl et al., 2013; Naderi et al., 2015). Both ESR1 and ESR2 play a role in estrogen-mediated neuroprotective function (Corder et al., 2004). In vitro studies have demonstrated that administration of ER agonists following toxic insults prevented neuronal cell death (Wise et al., 2001), and PPT and DPN have similar neuroprotective functions (Behl et al., 1995). ER agonists can elicit neuroprotective effects against various types of neuronal damage (Shao et al., 2012; Schreinhofer and Ma, 2013). Estrogen, via both ESR1 and ESR2, prevented microglial activation and thus exhibited neuroprotective function after neuronal injury (Jayaraman and Pike, 2009). Naderi et al. (2015) reported that type 1 and 2 specific receptor antagonists removed the neuroprotective effects of estrogen on brain edema and improved BBB disruption (Naderi et al., 2015). While all studies have shown that both ER agonists have a similar effect, one study reported that the effect of PPT was higher than DPN (Behl et al., 1995).

In the present study, it was also found that PPT and DPN caused both mRNA and protein level elevation of ESR1 and ESR2 after TBI. Consist with this study, it has been reported that receptor-specific agonists or estrogen exert some effects by altering the expression of type 1 and 2 receptor genes (Khaksari et al., 2015b). Studies have shown that gonadal steroids are involved in regulating ER protein levels in the brain (Chang et al., 2009; Gillies and McArthur, 2010). Also, a decrease in ESR2 expression (Westberry et al., 2008), and an increase in ESR1 were found to occur in female rats (Clipperton et al., 2008) after estrogen administration or cerebral ischemia. Administration of PPT and DPN in fibroblasts increased ESR1 and ESR2 expression at both the mRNA and protein level (Thakur and Sharma, 2007). PPT, DPN, or estrogen protected microglia from LPS toxicity by preventing cell death via upregulated ER expression (Smith et al., 2011). The probable mechanism(s) underlying ER agonist treatment at the transcriptional level of gene expression include regulation of DNA methylation (Wan et al., 2015), turnover of the protein (Thakur and Sharma, 2007), changes in the protein half-life, Esr1 and Esr2 mRNA levels (Crews et al., 2004), and PDZK-1 protein expression (Stossi et al., 2004).

The exact molecular signaling pathways that result in neuroprotective function still remain mostly unclarified. Recent analysis proposes that ESR1 and ESR2 protein expression levels play a key role in ER agonist prevention of neuroinflammation and eliciting neuroprotective functions in Alzheimer’s disease (Lee et al., 2014). It has been shown that stimulation of ERs has a promoting effect on PDZK-1 protein expression (Stossi et al., 2004). On the other hand, it has been demonstrated that PDZK-1 can stimulate the expression of genes in the cell nucleus (Stossi et al., 2004). Recently, it was reported that, in addition to the genomic responses of E2 mediated by classical estrogen receptors (ESR1 and ESR2), rapid non-genomic actions via the interaction of E2 with GPER occur (Alexander et al., 2017). Recent studies have demonstrated that GPER...
activation enhances protection against brain injuries (Lu et al., 2016). GPER has a high level of expression in both hippocampus and cortex (Brailoiu et al., 2007), while a GPER-selective antagonist reduces the neuroprotective effects of E2 (Gingerich et al., 2010; Roque et al., 2019). Indeed, GPER activation diminished inflammatory cytokines and blood-barrier permeability and enhanced neuronal survival in ischemic stroke animal models (Kosaka et al., 2012; Day et al., 2013; Lu et al., 2016; Zhao et al., 2016). Therefore, changes in receptor gene expression should be considered as a new therapeu- 

CONCLUSION

Our findings demonstrate that both ESR2 agonist DPN and ESR1 agonist PPT treatment can protect the brain against edema in a rodent model of TBI. This means that PPT and DPN can be therapeutic targets against the formation of edema after TBI. Furthermore, the upregulation of mRNA and protein levels of ESR1 and ESR2 after TBI may be another mechanism by which ER agonists induce antiedema effects because both PPT and DPN caused an increase in mRNA and protein levels of ESR1 and ESR2 after TBI. In addition, the effects of the combined group PPT+DPN on brain edema, BBB disruption, and ER protein level were higher than with the administration of either ER agonist alone. This means that both classical estrogen receptors might be involved in the neuroprotective action of E2. Based on the findings of the present study and previous investigations, evaluation of the combinatorial neuroprotective effects of PPT, DPN, and GPER is suggested for future experimental studies.

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