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Raloxifene protects against seizures and neurodegeneration in a mouse model mimicking epilepsy in postmenopausal woman

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ABSTRACT

Epilepsy in menopausal women presents several challenges in the treatment including an increased risk of seizures due to hormone replacement therapy. We investigated the hypothesis if raloxifene, a selective oestrogen receptor modulator, could be employed to prevent behavioural seizures and morphological alterations in a mouse model mimicking epilepsy in postmenopausal women. Female mice were made ovotoxic by treatment with 4-vinylcyclohexene diepoxide (VCD) to mimic a postmenopausal state. They were then subjected to kainic acid (KA)-induced seizures and neurotoxicity, as assessed by microscopic examination of hippocampus, relevant to human temporal lobe epilepsy. VCD administration (for 15 days followed by a drug-free period of 30 days) induced ovotoxicity in mice as evidenced by reduced number of primary ovarian follicles. This was accompanied by a 62.4% reduction in serum oestradiol levels. The bone mineral density of ovotoxic mice, however, remained unaffected. Raloxifene (8 mg/kg) reduced the seizure severity score in both normal and ovotoxic mice and protected against degeneration induced by KA in the CA3, CA1 sub-fields and hilus of the DG. Hippocampal TGF-β3 levels were not affected by any of the treatments. We show the potential protective role of raloxifene in preventing seizures and neuronal damage in a mouse model mimicking epilepsy in postmenopausal women which was found unrelated to hippocampal TGF-β3. Raloxifene might represent a novel therapeutic option for postmenopausal temporal lobe epileptic woman.

1. Introduction

Substantial evidence exists for the neuroprotective actions of the steroid hormone 17-β oestradiol (E2) (Behl and Manthey, 2000; Garcia-Segura et al., 2001). It has been shown that it prevented neuronal death from various insults including glutamate excitotoxicity (Hilton et al., 2006), ischemia (Guo et al., 2010), amyloid beta neurotoxicity (Marin et al., 2003) and protected hilar neurones from kainic acid (KA)-induced toxicity (Azcoitia et al., 1998; Hoffman et al., 2006). However, its use in epilepsy to prevent seizure-induced cell death is inadvisable because oestrogens are known to lower the seizure threshold and to facilitate seizures (Buterbaugh, 1989; Woolley, 2000; Reddy, 2009). Thus its use in postmenopausal women with epilepsy is imprudent even though oestrogen replacement after menopause is necessary in some women to protect against decline in verbal memory (Sherwin, 2002) or to decrease the progression of subclinical atherosclerosis (Hodis et al., 2001) or to maintain bone balance (Turner et al., 1994). Further, its use as hormone replacement therapy (HRT) is associated with various negative effects in the periphery such as proliferation of breast epithelium (Russo and Irma, 2006) and increased risk of endometrial adenocarcinoma (Gambrell et al., 1983). These limitations have been overcome by selective oestrogen receptor modulators (SERMs) which act as oestrogen agonist in bone, cardiovascular system and brain and as an antagonist in uterus and breast (Ibrahim and Hortobagyi, 1999) and thus have emerged as an alternative target for providing neuroprotection (Arevalo et al., 2011). Raloxifene (RLX), a SERM approved for osteoporosis, has been demonstrated to protect the hippocampus from the excitotoxic effects of KA (Ciriza et al., 2004) and has been shown to reduce morbidity, number of seizures and to delay the latency to seizures after pilocarpine-induced status epilepticus (SE) in rats (Scharfman et al., 2009).

Transforming growth factors beta form a small group of related proteins involved in the regulation of proliferation, differentiation, and survival of various cell types. Recently, exogenous TGF-β3 (5 or

Abbreviations: AED, antiepileptic drug; BMD, bone mineral density; DEXA, dual energy X-ray absorptiometry; DG, dentate gyrus; E2, 17-β oestradiol; HRT, hormone replacement therapy; KA, kainic acid; OVX, ovariectomy; RLX, raloxifene; SE, status epilepticus; SERMs, selective oestrogen receptor modulators; TGF, transforming growth factor; TLE, temporal lobe epilepsy; VCD, 4-vinylcyclohexene diepoxide.

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10 ng/i.c.v.) was found to significantly attenuate KA-induced seizures and neuronal damage in rats. SE activated TGF-β signalling and the same appeared to be a potential target for preventing epileptogenesis and secondary damage following SE (Kim et al., 2002).

Ovariectomy (OVX) is the most commonly employed animal model for studying the effect of ovarian steroids on brain function (Schauwecker et al., 2009). However, OVX causes a sudden decline in oestrogen and eliminates all ovarian secretions (Schauwecker et al., 2009). Mayer et al. (2002) reported that the industrial chemical 4-vinylcyclohexene diepoxide (VCD) selectively kills the small primordial and primary ovarian follicles leading to a gradual decline in reproductive activity and is considered to model menopause in rodents.

Since KA-induced SE is known to produce behavioural and histopathological features resembling mesial temporal lobe epilepsy (TLE) in humans (Ben-Ari, 1985), we envisaged to mimic a state representing epilepsy in postmenopausal women by subjecting VCD-induced ovotoxic mice to KA-induced SE and neurotoxicity. The effect of RLX in this model and on hippocampal TGFbeta3 levels was assessed in ovotoxic mice.

2. Experimental procedures

2.1. Animals

Adult Swiss albino female mice (25–35 g), procured from the Central Animal House Facility of Hamdard University, New Delhi, were used. The animals were housed in polypropylene cages under natural light/dark cycle and controlled conditions of temperature and humidity (25 ± 2 °C, 55–65%). They were fed commercial pellet diet (Amrut rat and mice feed, Chakan Oil Mills, Pune, India) and water *ad libitum*. At least a day before performing the experiment, each mouse was acclimatised to the laboratory conditions. All experimental procedures strictly complied with the guidelines of the Institutional Animal Ethics Committee of Jamia Hamdard, New Delhi (file no 920 for the year 2013).

2.2. Drugs, dosing schedule and experimental design

VCD and KA were procured from Sigma–Aldrich (India) and RLX from Dr. Reddy's laboratories, Hyderabad. Drug solutions were prepared in pyrogen-free sterile water for injection. VCD was dissolved in sesame oil and raloxifene was suspended in 1% CMC. All treatments were injected intraperitoneally (i.p.) in a volume not exceeding 10 ml/kg. RLX was administered in doses of 2, 4 and 8 mg/kg for a period of 15 days prior to KA administration in ovotoxic mice. The dose of 8 mg/kg was derived from the corresponding dose (60 mg) in humans for the treatment of osteoporosis (Delmas et al., 2002).

Fifty-four mice were randomly selected and divided into nine experimental groups with six animals each group. First three groups of mice were left non-ovotoxic and were treated with Group 1: Saline (normal control), Group 2: KA (10 mg/kg), Group 3: RLX (4 mg/kg) + KA. Remaining six groups of mice underwent treatment with VCD to induce ovotoxicity, including, Group 4: VCD (ovotoxic control), Group 5: VCD + KA, Groups 6, 7 and 8 received RLX 2, 4 and 8 mg/kg, i.p. respectively followed by KA in VCD treated ovotoxic mice and Group 9 received RLX (8 mg/kg) in ovotoxic mice.

2.3. Induction of ovotoxicity

VCD was administered at a dose of 320 mg/kg i.p. for 15 days followed by a drug-free period of 30 days to induce ovotoxicity (Schauwecker et al., 2009). After 45 days, one animal each from the normal control and ovotoxic control group was euthanized with ether followed by removal of ovaries and subjected to histopathological evaluation. Ovarian damage was reported as comparative presence of follicles in two groups. In addition, blood samples were taken from three animals of each group for measurement of serum oestradiol levels using a commercially available ELISA kit (manufactured by CusaBiotech bearing Catalog no: CSB-E05109m).

2.4. Bone mineral density

Lumbar vertebrae and femoral bones were removed from normal and ovotoxic mice for measurement of bone mineral density (BMD) which was performed using DEXA Fan Beam Densitometer Model, Discovery A (S/N 84023), Hologic, USA at AIIMS, New-Delhi. Scans of isolated bones were performed at a scan speed of 1 mm/s or 4 lines/mm.

2.5. KA-induced behavioural seizures

After confirming ovotoxicity, mice were administered KA (10 mg/kg i.p.) to induce SE (as per Huang et al., 2009). This dose was also standardised in our laboratory to induce low-grade or mild seizures (stages 0–4) in all the animals without mortality in preliminary experiments. Animals were then monitored for 120 min to assess the severity and length of behavioural seizures. The behavioural progression of KA-induced seizures intensity was scored with a slight modification from Racine classification (1972) as follows: Stage 1: immobilization and staring, Stage 2: head nodding, Stage 3: rearing accompanied by forelimb clonus and wet dog shakes, Stage 4: falling and wobbling, Stage 5: jumping, circling, or rolling, Stage 6: severe tonic–clonic seizures. Seizure grades 1–4 were regarded as low-grade or mild seizures and 5–6 as high-grade or severe seizures. A score was recorded when the length of a particular stage sustained for $\geqslant$4 s (Kim et al., 2010).

2.6. KA-induced neuronal damage

KA-induced neuronal damage was assessed by histopathological evaluation of hippocampus on 7th day post KA administration. Animals from each group were deeply anesthetised with ether, brains removed from the skull and preserved in formalin solution. Brains were cut in serial coronal (10 μm) sections through the hippocampus on a microtome (VT1000S; Leica, Wetzlar, Germany). Hippocampal slides were made out of them and haematoxylin–eosin staining was used to assess neuronal, cellular and terminal degeneration. Neuronal damage or loss was assessed by determining the frequency of pyknotic cells in CA1, CA3 and DG regions of the hippocampus when observed under optical microscope (Olympus BX 50, Japan). The total score for each animal was obtained by summing the scores of all three sections. The neuronal loss has been shown as the percentage of pyknotic cells (total number of pyknotic cells/total number of intact cells × 100) representing each treatment group.

2.7. Estimation of hippocampal TGF-β3

Supernatants from tissue homogenate in RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM EDTA) were removed and assayed in duplicate according to the manufacturer's instructions. Levels of TGF-β3 in the hippocampal tissue was determined using a commercially available ELISA kit manufactured by Cusa Biotech bearing Catalog no: CSB-E12862m.