Journal of Steroid Biochemistry & Molecular Biology 116 (2009) 1–7

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Estrogen receptors increased expression during hippocampal neuroprotection in lactating rats

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ARTICLE INFO

Article history:
Received 3 October 2008
Received in revised form 10 February 2009
Accepted 12 February 2009

Keywords:
Estrogen receptor
Neuroprotection
Hippocampus
Lactation

ABSTRACT

Estrogen receptor (ER)-mediated neuroprotection has been demonstrated in both *in vitro* and *in vivo* model systems. Two types of estrogen receptors, ERα and ERβ, are the major mediators of the biological functions of estrogens. In the hippocampus, ERβ is prevalent over ERα. Recently, we reported that during the final phase of lactation there is a neuroprotective mechanism in the hippocampus of the adult female rat against neuronal damage induced by systemic kainic acid administration vs. virgin (metestrus) rats. In this study, we assessed differential ER expression and localization in CA1, CA3 and dentate gyrus regions of dorsal hippocampus of metestrus and lactating adult rats at day 19 of lactation, during basal conditions (metestrus and L19, respectively) and 24 h after systemic kainate administration. ERs were assessed by western blot and immunohistochemistry. We found a significant increase in the expression of ERs in the hippocampus during lactation as compared with metestrus. ERβ was significantly increased in the CA1 and CA3 of lactating rats after the kainic acid insult. In addition, we observed a relocalization of ERβ from the cytoplasm to the nucleus of neuronal cells. Our results suggest that there is a strong correlation between expression of ERs, especially ERβ, in lactating CA1 and CA3 hippocampus regions in response to kainate administration, and neuroprotection observed during this reproductive period. This may be one of the mechanisms involved in the protection of the maternal brain to ensure offspring survival.

1. Introduction

Estrogens modulate gene expression in different tissues, the more studied effects are found in the reproductive tract and in the central nervous system (CNS) [1,2]. Estrogens exert their effects through two subtypes of estrogen receptors (ERs), ERα and ERβ. ERs belong to the nuclear receptor superfamily and are widely distributed in the brain and spinal cord [2].

Estrogens and their receptors can activate cell functions via genomic and non-genomic mechanisms. The genomic mechanism requires a series of steps such as binding of estrogens to the receptor, translocation of ERs into the nucleus, dimerization, and interaction with specific estrogen receptor binding sites (ERE) on promoters of estrogen regulated genes. The indirect or non-genomic mechanisms consist of the activation of different signaling pathways including mitogen activated protein kinases (MAPK), Akt, cAMP-responsive element binding protein (CREB) and regulation of intracellular ion concentration through ion channel modulation, and Ca^{2+} homeostasis. In addition, they can also modulate anti-apoptotic genes, such as members of the Bcl-2 family of proteins and caspases [3–6].

Estrogen receptors mediate different effects on the structure and function of the central nervous system, including excitability, neurotransmitter release, memory processes, spine density, synapsis, neurogenesis and neuroprotection [7–14]. In the rodent brain, estrogen receptors have been identified by autoradiography assay using [^{3}H]estradiol [15]. High expression of ERs was found in different limbic system regions such as the hippocampus, amygdala and lateral septum. These findings were later confirmed using immunohistochemical and *in situ* hybridization techniques [16,17].

It has been accepted that estrogens, through ERs, participate in neuroprotection in different neurodegeneration models and ischemia [18,19]. The contribution of ERs to neuroprotection has been related to transcriptional regulation of proapoptotic and anti-apoptotic molecules [20].

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doi:10.1016/j.jsbmb.2009.02.015

Recently, we reported that during lactation there is a neuroprotective effect against cell damage induced by kainate in dorsal hippocampus. Using different methods to assess cell damage, we established that the brain of the lactating rat was significantly less sensitive to excitotoxic damage induced by kainate than the metestrus rat [21]. The aim of the present study was to determine changes in the expression of ERs and their distribution in the hippocampus of normal and kainic acid treated lactating rats, to elucidate the possible contribution of ERs in neuroprotection against kainic acid cell damage observed in lactating rats.

2. Materials and methods

2.1. Animals

Adult virgin or pregnant (18–20 days) female Wistar rats (250–300 g) were housed individually under controlled temperature and lighting conditions (12:12 h light:dark cycle, lights on at 06:00 h), with food and water available *ad libitum*.

Lactating rats at the final phase of lactation were chosen for this study based on the expected steroid hormone levels in comparison to metestrus rats. Similar levels of sexual steroid hormones, estradiol (10.5 ± 6.3 pg/ml vs. 15.0 ± 5.7 pg/ml) and progesterone (<10.0 ng/ml) are exhibited under both conditions, but circulating levels of corticosterone (>300 ng/ml in lactating vs. <300 ng/ml in metestrus rats) are chronically higher during lactation [21,22]. Each experimental or control group contained five animals.

For lactating rats, 1 day after parturition, litter sizes were culled to 8–10. Mothers were kept undisturbed with the litters, and they were used for experiments on lactating day 19, considered as the basal condition. Treated animals received an intraperitoneal (i.p.) injection of vehicle (PBS) or kainate (7.5 mg/kg) and were sacrificed 24 h later.

Vaginal smears of virgin female rats (200–250 g) were followed for at least four cycles. Metestrus was considered to be the basal condition. Animals on this day of the oestrous cycle received the same treatment with vehicle (PBS) or KA (7.5 mg/kg) as above, and were perfused 24 h later.

The Institutional Animal Care and Use Committees of the School of Chemistry and Institute for Neurobiology at the National Autonomous University of Mexico approved all experimental protocols. Animals were handled in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Official Mexican Guide of the Ministry of Agriculture (SAGARPA NOM-062-Z00-1999) published in 2001 [http://www.sagarpa.gob.mx/ganaderia/NOM/029zoo.pdf]. All efforts were made to minimize the number of animals used and their suffering.

2.2. Western blot

Dorsal hippocampal regions, CA1, CA3 and dentate gyrus (DG), from lactating and virgin animals were dissected according to the Lein [23] procedure. Tissue was homogenized by sonication (four cycles at 100% of amplitude during 20 s) in 200 μl of lysis buffer. Homogenates were centrifuged at 12,500 rpm during 15 min at 4 °C and supernatants recovered. Thirty μg of total protein was separated by 8% SDS-PAGE for ERα and ERβ detection. Following transfer onto PVDF membranes, blots were blocked with 10% dry milk in TBS-0.1% Tween and incubated overnight at 4 °C in primary antibodies against ERα (1:400, Santa Cruz Biotechnology), ERβ (1:500, Santa Cruz Biotechnology) and β-actin (1:500, Santa Cruz Biotechnology) followed by horseradish peroxidase (HRP) conjugated secondary anti-rabbit or anti-mouse secondary antibodies (1:10,000, Santa Cruz Biotechnology). Immunoreactivity was visualized using enhanced chemiluminescence. Data were expressed as ratios of β-actin.

2.3. Tissue preparation

Rats were deeply anesthetized with pentobarbital (Sigma–Aldrich, St. Louis, MO) to carry out transcardiac perfusion with 250 ml of 0.1 M PBS (pH 7.4) followed by 250 ml of 4% paraformaldehyde in PBS (pH 9.5, 10 °C). Brains were removed, postfixed in the same fixative overnight and cryoprotected using 20% sucrose for 2–3 days at 4 °C. Coronal sections (30 μm) were cut through the dorsal hippocampus on a freezing microtome, and six series were collected and stored in cryoprotectant solution (30% ethylene glycol and 20% glycerol in PBS) at −20 °C. One of the six series was employed for each staining, such that consecutive slices of tissue were analyzed by the different methods. Before any procedure, free-floating sections were rinsed 3 times for 10 min in PBS buffer. The sections were mounted on poly-lysine slides, dried, and kept in PBS overnight to eliminate residual cryoprotectant.

2.4. Immunohistochemistry

Immunoreactivity for estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ) was detected using a conventional avidin–biotin–immunoperoxidase technique [24]. Tissue was labeled with cell-type-specific polyclonal rabbit antibody against ERα (1:100, Santa Cruz Biotechnology, CA), or mouse monoclonal antibody against ERβ (1:100, Santa Cruz Biotechnology,

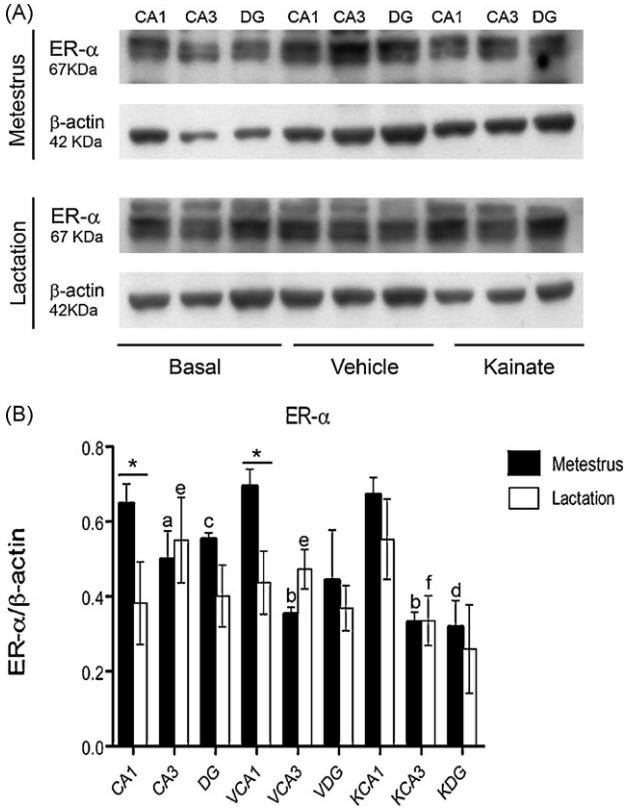

Fig. 1. ERα expression in the hippocampus. (A) ERα expression was measured by western blot in CA1, CA3 and DG hippocampal regions of virgin and lactating rats during basal conditions (metestrus and lactation day 19, respectively) and 24 h after vehicle (V) or kainate (K) administration. A representative western blot of five independent experiments is shown. (B) Western blot densitometric analysis. Data represent the mean ± S.D. of five animals/treatment. Metestrus vs. lactation: $^{*}p < 0.001$; a vs. b: $p < 0.01$; c vs. d: $p < 0.05$; e vs. f: $p < 0.05$.

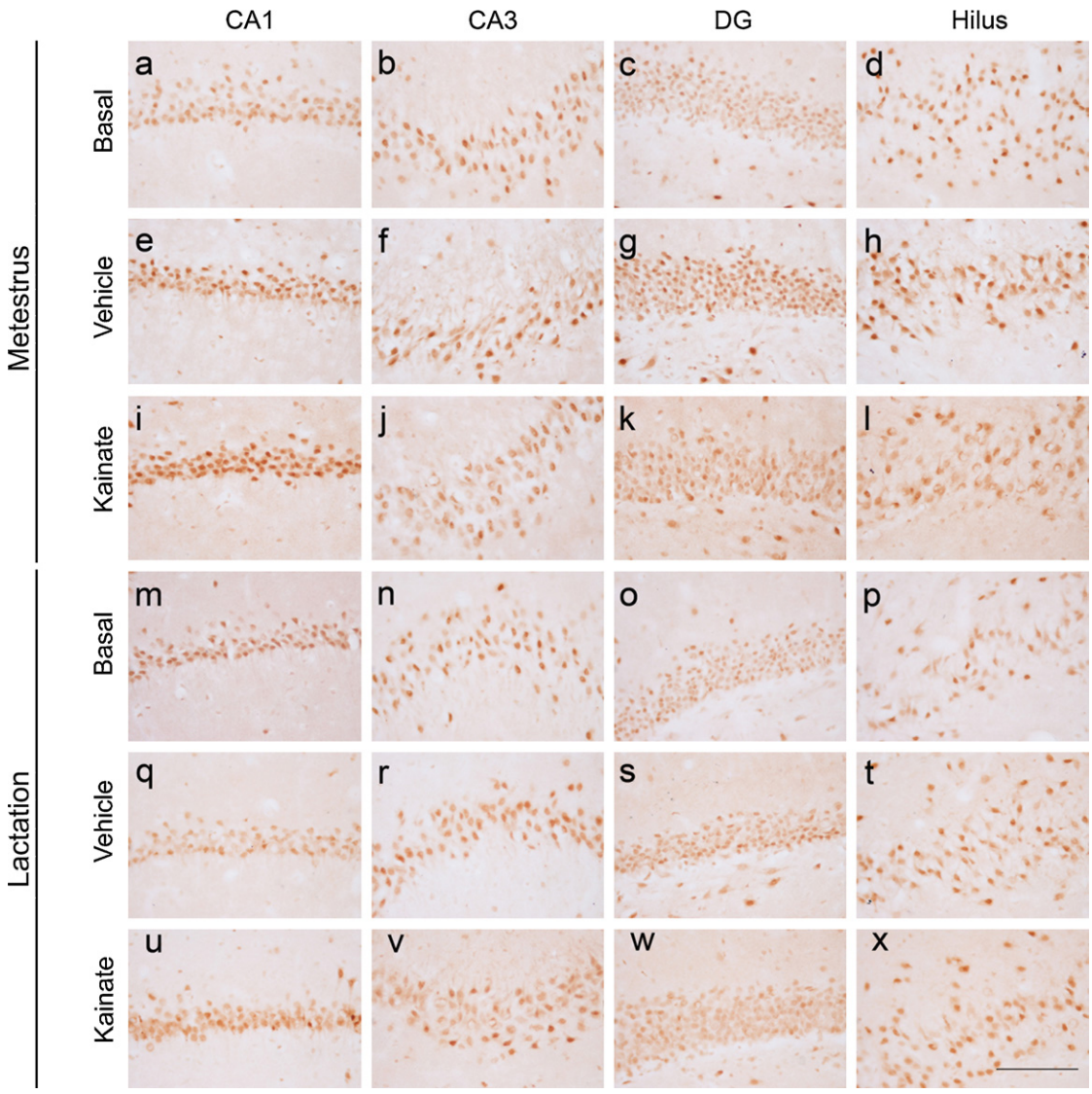

Fig. 2. ERα cellular localization in the hippocampus. ERα protein was detected *in situ* by immunohistochemistry in dorsal hippocampus of virgin metestrus (a–l) and lactating (m–x) rats, in basal conditions (a–d; m–p), and after vehicle (e–h; q–t) or kainate (i–l; u–x) administration, in pyramidal cells of CA1 region (a, e, i, m, q and u) and CA3 stratus (b, f, j, n, r and v) and granular cells of DG (c, g, k, o, s and w) and hilus region (d, h, l, p, t and x). ERα is preferentially located in the nuclei of neurons. Bar = 100 μm.

CA). Sections were mounted and dried on poly-L-lys-treated slides and treated with boiling sodium citrate 10 mM pH 6.0 during 20 min, 3% hydrogen peroxide for 30 min to quench endogenous peroxidase activity, 1% sodium borohydride in PBS at 4 °C for 30 min, Triton X-100 0.3% in PBS for 30 min with several washes in PBS. The tissue was then incubated with blocking solution (5% BSA/2% goat or rabbit serum/1% Triton X-100 in PBS) for 1 h to decrease non-specific labeling.

Sections were incubated with primary antibody in blocking solution at 4 °C overnight. After washing, primary antibody was detected with the biotinylated secondary antibody and the avidin/biotin system (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA). Sections without primary antibodies or with primary antibodies preadsorbed with the corresponding peptide were processed in parallel as negative controls.

2.5. Statistical analysis

Western blot densitometric results for each group are presented as mean ± S.D. Statistical significance between groups was established with a one-way ANOVA followed by the Bonferroni multiple comparisons test. $p < 0.05$ was considered significant.

Positive cell nuclei for ERβ immunolabeling count were performed with microscopic images obtained from pyramidal cell layers in CA1 and CA3 and hilus and granular cell layers in DG subdivisions of the hippocampus using 40× magnification. Statistical significance between groups was established with a one-way ANOVA followed by the Bonferroni multiple comparisons test. $p < 0.05$ was considered significant.

3. Results

3.1. Cellular localization and expression assessment of ERα in hippocampal areas

The level of ERα in total protein extracts from dorsal hippocampal regions was analyzed by western blot (Fig. 1A). The results showed that during metestrus, there was an increased expression of ERα in the CA1 region as compared with the CA3 region. Decreased expression of ERα was observed in CA3 and DG hip-

pocampal regions 24 h after systemic kainic acid administration (Fig. 1B).

Lactating rats on day 19 showed a similar expression of ERα to the metestrus rats in CA3 and DG hipocampal regions, while the CA1 region showed a lower expression of the receptor (Fig. 1A and B). Vehicle treatment did not affect ERα expression. After kainate treatment, we observed a significant decrease in ERα expression on the CA3 region as compared with basal conditions and vehicle treatment (Fig. 1B).

To assess ERα distribution in the hippocampus, we employed an immunohistochemical approach. ERα was detected at high levels in the nuclei of pyramidal neurons of the CA1 and CA3 regions and the DG granular and hilar neurons in virgin animals during basal conditions (Fig. 2a–d). After 24 h of vehicle administration, an increase in cytoplasmic ERα in the CA1 and CA3 pyramidal neurons and hilar neurons was observed in the hippocampus of diestrus rats, where immunoreactivity extends into axonal projections of the neurons (Fig. 2e–h). This change, from preferentially nuclear to nuclear–cytoplasmic distribution of ERα, was prevented by kainate treatment. The hippocampus of metestrus animals, 24 h after systemic administration of kainic acid, showed a preferential nuclear pattern of ERα distribution, similar to basal conditions (Fig. 2j–l).

Hippocampal regions of lactating animals, in basal conditions (lactating day 19), showed ERα immunoreactivity in pyramidal neuron nuclei of the CA1 and CA3 regions and in the nuclei of granular and hilar neurons of the dentate gyrus, with slight reactivity in the cytoplasm (Fig. 2m–p). The nuclear–cytoplasmic distribution pattern of ERα in lactating rats shows no change after vehicle (Fig. 2q–t) or kainate administration (Fig. 2s–x).

3.2. Cellular relocalization and expression changes of ERβ in hippocampal areas after kainic acid administration to lactating rats

Expression of ERβ was measured in total protein extracts from dorsal hippocampal regions by western blot (Fig. 3A). Metestrus rats showed low expression levels in the CA1 hippocampal region as compared with the CA3 and DG regions under basal conditions. After 24 h of vehicle administration, on diestrus day of the oestrous cycle, we observed an increase in ERβ expression in the CA1 region and a decrease in the DG region, whereas ERβ expression in the CA3 region was not modified. The systemic administration of kainic acid induced a decrease in ERβ concentration in the CA1 region while an increase in ERβ expression was observed in the DG region (Fig. 3A and B).

During lactation, ERβ protein in the hippocampus showed similar levels to those during metestrus, as detected by western blot, in all the studied areas. Vehicle administration did not affect ERβ expression in the CA1, CA3 and DG regions of lactating rats. After 24 h of kainic acid administration, a significant increase in ERβ protein levels was detected in the CA1 and CA3 dorsal hippocampal regions (Fig. 3A and B).

Localization of ERβ in dorsal hippocampus in virgin and lactating rats was visualized by immunohistochemistry. Virgin rats on metestrus day showed a marked perinuclear localization of ERβ protein in CA1 and CA3 pyramidal neurons. Additionally, immunoreactivity was positive in interneuron strata (data not shown). In the dentate gyrus, ERβ expression showed perinuclear immunoreactivity both in granular and hilar neurons (Fig. 4a–d). Treatment with vehicle or kainate did not affect perinuclear distribution of ERβ protein (Fig. 4e–l and 5A).

The hippocampus of lactating rats under basal and vehicle conditions showed perinuclear distribution in the soma of pyramidal, granular and hilar neurons of the CA1, CA3 and DG regions (Figs. 4m–t and 5A). Immunoreactivity was positive for axonal projections in the principal strata of the hippocampus. In addition,

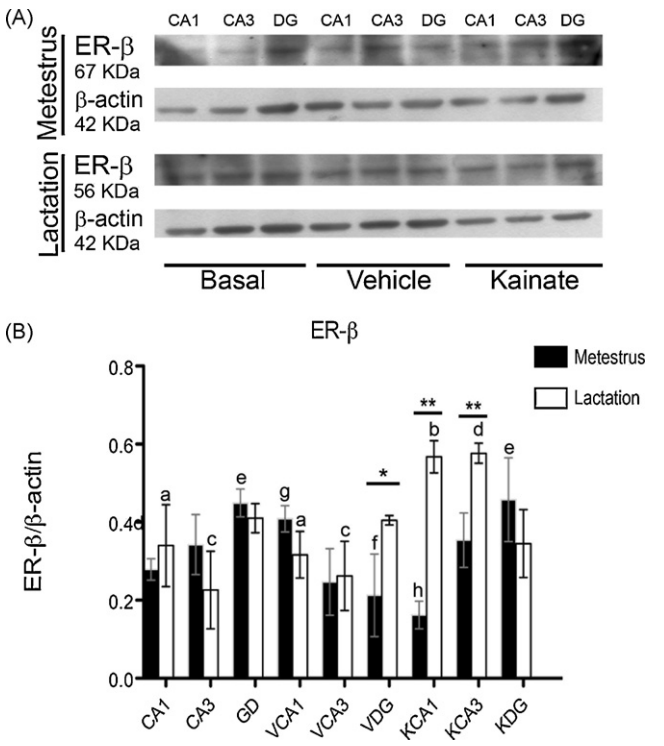

Fig. 3. ERβ expression in the hippocampus. (A) ERβ expression was measured by western blot in CA1, CA3 and DG hippocampal regions of virgin and lactating rats during basal conditions (metaestrus and lactation day 19, respectively) and 24 h after vehicle (V) or kainate (K) administration. A representative western blot of five independent experiments is shown. (B) Western blot densitometric analysis. Data represent the mean ± S.D. of five animals/treatment. Metestrus vs. lactation: $^{*}p < 0.05$, $^{**}p < 0.001$; a vs. b: $p < 0.05$; c vs. d: $p < 0.01$; e vs. f: $p < 0.05$; g vs. h: $p < 0.01$.

we observed ERβ in interneuronal strata (data not shown). Kainic acid systemic administration dramatically changed the distribution of ERβ in CA1, CA3 and DG regions (Fig. 5A). About 30% of the cells in the principal strata of hippocampal regions showed cytoplasmic localization of ERβ before kainate treatment (Fig. 5B). After 24 h of kainate administration, the principal strata of the hippocampus showed a redistribution of ERβ protein from cytoplasmic localization to a nuclear–cytoplasmic distribution in about 75% of pyramidal granular and hilar neurons (Figs. 4u–x and 5).

4. Discussion

In the present study we demonstrate that estrogen receptor beta expression is significantly increased in the CA1 and CA3 regions of the hippocampus in lactating rats after treatment with kainic acid, but not in the DG. In contrast, the expression of estrogen receptor alpha decreased significantly after kainic treatment in CA3, in both metestrus and lactating rats, and in the DG of the metestrus rats. In addition, a signicant relocalization of ERβ from the cytoplasm to the nucleus was observed in hippocampal neurons, suggesting the participation of ERβ in neuroprotection against kainic acid cell death induction in this brain area.

Recently, we reported that the dorsal hippocampus of the mother is protected against cellular damage caused by systemic administration of KA, as compared to virgin rats during metestrus phase of the oestrous cycle, and the mechanisms that participate in this process are not fully understood [21]. However, the hormones involved in lactation, such as steroid hormones, are thought to participate in this phenomenon.

Given that contribution of both estrogen receptor subtypes to neuroprotection in the hippocampus has been demonstrated

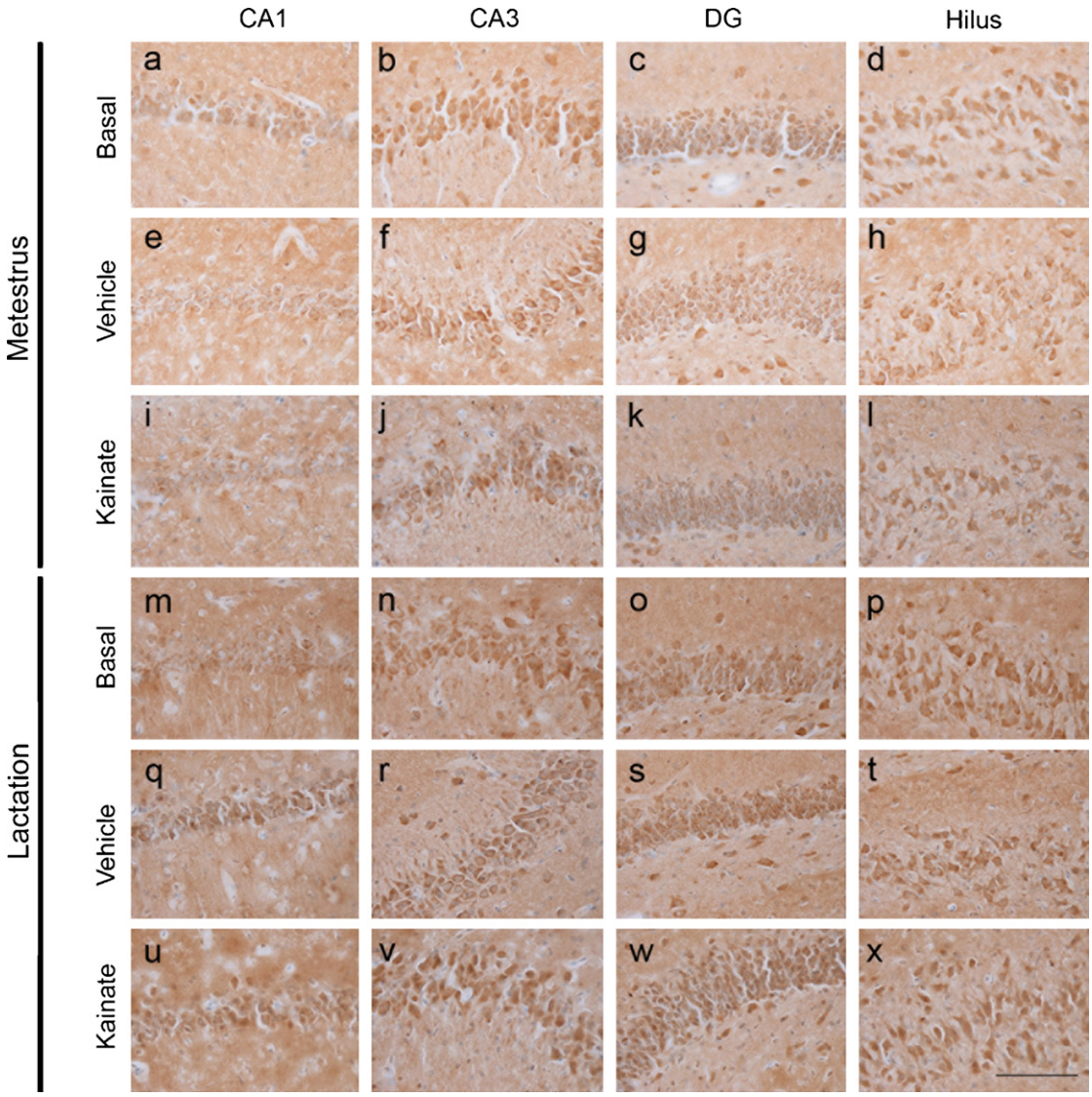

Fig. 4. ERβ cellular localization in the hippocampus. ERβ protein was detected *in situ* by immunohistochemistry in dorsal hippocampus of virgin (a–l) and lactating (m–x) rats, under basal conditions (a–d; m–p), and after vehicle (e–h; q–t) or kainate (i–l; u–x) administration, in pyramidal cells of CA1 (a, e, i, m, q and u) and CA3 stratus (b, f, j, n, r and v) and granular cells of DG (c, g, k, o, s and w) and the hilus region (d, h, l, p, t and x). ERβ was found in a perinuclear pattern in virgin rats while in lactating hippocampus, ERβ immunoreactivity was detected in the perinuclear region and in axonal projections of pyramidal and granular neurons. ERβ was detected in interneuron strata. The localization pattern of ERβ in virgin rats was not affected by treatments. In contrast, in the hippocampus of the lactating rats, a relocalization of ERβ was observed from a perinuclear pattern to a nuclear and cytoplasmatic distribution after kainate treatment. Bar = 100 μm.

[20], in the present study we sought to evaluate the changes in the expression of estrogen receptors alpha and beta in order to determine their contribution to the neuroprotection observed in lactating animals against kainic acid cell death induction.

Our finding that ERβ was significantly increased in hippocampal areas after kainate administration is on-line with previous observations about the important role of this receptor in this brain region. Although both ERα and ERβ are involved in mechanisms leading to estrogen-inducible neuronal responses, it has been demonstrated that they exhibit different signaling responses. The recent and important work of Zhao and Brinton, demonstrated that there is a greater potentiation of physiological glutamate-induced increase in intracellular Ca^{2+} exerted by an ERβ-selective agonist, suggesting that ERβ may be more involved than ERα in promoting mechanisms that modulate estrogen-inducible neuronal morphological plasticity and memory function [25]. This finding is also consistent with a number of *in vivo* animal observations, which demonstrate the important role of ERβ in regulating neurogenesis and brain development [26–28].

In other brain injury models, such as ischemia, it has been demonstrated that estrogen receptors mediate neuroprotection. Interestingly, Cimarosti et al. [29] reported changes in the expression of ERα and ERβ in rat organotypic hippocampal slice cultures treated with estradiol and subsequently exposed to oxygen–glucose deprivation (OGD). The levels of ERα protein were significantly reduced after OGD in both vehicle- and estradiol-treated cultures, whereas ERβ was significantly up-regulated in the estradiol-treated cultures. These findings suggest that estrogen-induced neuroprotection against ischemia might involve regulation of ERβ, and consequently, of the genes influenced by this receptor. In a very similar way, in the present study we found that while ERα presented only small changes in expression after treatment with kainic acid in the hippocampus of normal and lactating rats, ERβ was significantly up-regulated in the CA1 and CA3 regions of the hippocampus of lactating rats after kainic acid exposure, highlighting the importance of this protein in neuroprotection processes. Furthermore, the major finding of the present work was the fact that ERβ presented a relocation from cytoplasm to the nucleus in different areas of the

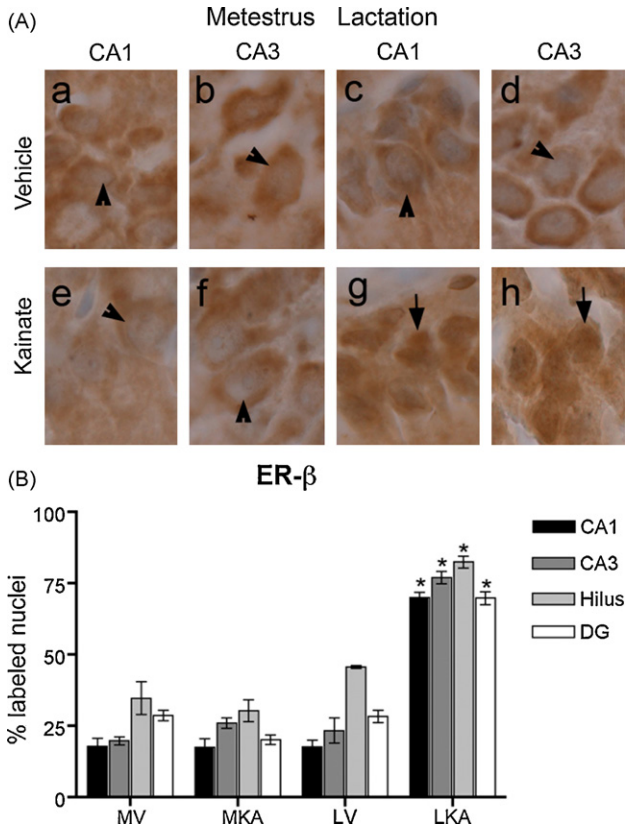

Fig. 5. Relocation of ERβ in hippocampal neurons. (A) Hippocampus of metestrus rats showed preferential cytoplasmic localization of ERβ protein in the principal strata of hippocampal regions (a and b), and the treatment with kainate did not affect distribution of the ERβ protein (e and f). The hippocampus of lactating rats showed cytoplasmic distribution in the soma of pyramidal, granular and hilar neurons of the CA1, CA3 and DG regions (c and d). Interestingly, positive neurons for nuclear ERβ immunoreactivity increased significantly after 24 h of kainic acid administration (g and h). (B) Percentage of positive neurons for nuclear ERβ immunoreactivity in CA1, CA3, hilus and DG regions of metestrus (M) and lactating (L) rats. Vehicle (V) vs. kainate (KA) treatment: $^{*}p < 0.001$. Arrowheads indicate cells with cytoplasmic staining; arrows indicate cells with nuclear staining.

hippocampus in the lactating rats after kainic acid administration, indicating the possibility that ERβ may acquire some new functions during excitotoxicity (Fig. 5).

Furthermore, other studies on estrogen-mediated neuroprotection in the hippocampus of post-ischemic adult macaque monkeys showed that ERα was present in control CA1 pyramidal neurons, decreasing after ischemia. In contrast, ERβ immunoreactivity increased remarkably in the radiate and molecular layers of the CA1, where it was present in astrocytes and microglia. ERα was negligible in both control and post-ischemic monkeys. These results suggest that ERβ is the major receptor responsible for the direct estrogen actions on the monkey hippocampus [30].

The Bcl-2 protein family has been implicated in the regulation of apoptosis [31]. Bcl-2 is the main antiapoptotic protein, and it has been shown to be involved in maintaining cellular viability in the brain [32]. Early observations about the role of Bcl-2 expression in experimental brain injury models were described by Nakamura [33]. The neuroprotective actions of ERα and more recently of ERβ in neuroprotective models include regulation of Bcl-2 protein expression [34,35]. Recent results from our group showed increased Bcl-2 immunolabeling in CA1 in the hippocampus, cerebral cortex (Ctx), and piriform cortex (Pyr ctx) in lactating rats suggesting that Bcl-2 protein participates in the protection against damage induced by KA [21]. However, more studies are necessary to confirm this observation and to determine the role of estrogens in this pathway.

The overall results of our study indicate that changes in the expression and relocalization of ERβ from the cytoplasm to the neuronal nucleus, occurring during excitotoxicity by kainic acid treatment in lactating rats, may be involved in neuroprotection observed in the hippocampus, and further support the idea that this receptor plays a major role in neuroprotection in this brain region.

Acknowledgements

This work was performed with partial financial support from, The National Council of Science and Technology (CONACyT), and PAPIIT, PAIP programs from the National Autonomous University of Mexico, Mexico City, Mexico.

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