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Estrogen modulates Bcl-2 family protein expression in the sexually dimorphic nucleus of the preoptic area of postnatal rats

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Abstract

In the sexually dimorphic nucleus of the preoptic area (SDN-POA) of postnatal rats, apoptotic cells are detected more frequently in females than males. This sex difference is under the influence of aromatized androgen. We have reported that there are sex differences in the levels of Bcl-2 (female < male) and Bax (female > male) in the central division of the medial preoptic nucleus (MPNc), a significant component of the SDN-POA, followed by a sex difference in induction of apoptosis via caspase-3 activation (female > male). In the present study, we examined effects of estradiol benzoate (EB) on expression of Bcl-2 and Bax in the MPNc. Female rats were subcutaneously injected with EB (25 or 50 μg per head) on postnatal day 5. MPNc and caudate putamen (CP) tissues were obtained from EB-treated female and male rats on postnatal day 6. Protein levels of Bcl-2 and Bax were determined by Western blotting. In the MPNc of female rats, EB at a dose of 50 μg/head but not 25 μg/head significantly increased Bcl-2 protein level and decreased Bax protein level. The levels of Bcl-2 and Bax of female rats treated with 50 μg of EB were comparable to those of male rats. However, the protein levels of Bcl-2 and Bax in the CP did not change with EB treatment. These results suggest that estrogen up-regulates Bcl-2 expression and down-regulates Bax expression in the MPNc of postnatal rats. Effects of estrogen on the Bcl-2 family are presumably responsible for sex difference in postnatal apoptosis of the SDN-POA.

Keywords: Estradiol; Apoptosis; Bcl-2; Bax; Sexually dimorphic nucleus of the preoptic area (SDN-POA)

Morphological sex differences in the central nervous system are well documented. In rats, the sexually dimorphic nucleus of the preoptic area (SDN-POA) of males is larger and has more neurons than that of females [12,13]. The volume of the SDN-POA in male rats decreases when they are neonatally castrated [12]. In contrast, in female rats that receive testosterone propionate (TP) treatments in the perinatal period, the SDN-POA enlarges until it is as large as that of normal males [7,12]. Thus, the gonadal steroidal milieu during the perinatal period is closely related to establishment of sexual dimorphism of the SDN-POA.

Although the mechanisms involved in formation of the sexually dimorphic nuclei are unclear, several lines of evidence indicate that they involve sex differences in the number of neurons killed by apoptosis during brain development. In the central division of the medial preoptic nucleus (MPNc), which is a significant component of the SDN-POA, the number of apoptotic cells is greater in female rats than in male rats in the postnatal period [4,5]. The sex difference in the apoptotic cell number of the MPNc in the postnatal period negatively correlates with the sex difference in the volume and cell number in adulthood. In addition, in neonatally gonadectomized female and male rats, the number of apoptotic cells in the MPNc in the postnatal period is decreased by TP treatment [4,5]. Furthermore, in postnatal female rats, estradiol benzoate (EB) reduces the number of apoptotic cells in the SDN-POA [2]. Together, these findings suggest that estradiol, which is synthesized from testosterone by aromatase in the brain, inhibits apoptotic cell death in the SDN-POA of postnatal rats.

The Bcl-2 family, which is involved in regulation of cell death and survival via the mitochondrial apoptotic pathway, plays an important role in the formation of the sexually dimorphic nuclei [8]. The anteroventral periventricular nucleus (AVPV) of male mice and the spinal nucleus of the bulbocavernosus (SNB) of female mice normally have fewer cells than those of the opposite sex, but overexpression of anti-apoptotic Bcl-2 increases the cell numbers of these nuclei and reduces the sex differences in their cell numbers [24]. In addition, in pro-apoptotic Bax knockout mice, morphological sex differences in the AVPV, SNB, and

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the principal nucleus of the bed nucleus of the stria terminalis (BSTp) are reduced or absent [9,16].

We previously reported that, in the MPNc of postnatal rats, there are sex differences in the expression levels of Bcl-2 (female < male) and Bax (female > male) and in the number of neurons containing active caspase-3 (female > male) [21]. These findings suggest that, in the SDN-POA of postnatal rats, the sex difference in the number of apoptotic cells is caused by a sex difference in the apoptotic signal cascade that involves Bcl-2, Bax and caspase-3. However, it is unclear whether estradiol modulates this apoptotic signal cascade in the SDN-POA of postnatal rats. In the present study, we investigated the effects of EB on expression of Bcl-2 and Bax in the SDN-POA of postnatal female rats.

Pregnant Long-Evans rats were purchased as breeding stock (Institute for Animal Reproduction, Kasumigaura, Japan), and were bred with a 12-h light/12-h dark cycle at 23 ± 1 °C with free access to water and food. Female and male pups delivered by the breeding stock were used in the present experiments. All experiments were conducted according to the Guidelines for the Care and Use of Experimental Animals of the National Institute of Environmental Studies.

On postnatal day 5 (PD5; day of birth = PD1), female pups were subcutaneously injected with 25 or 50 μg of EB dissolved in 0.05 ml corn oil (EB25 and EB50 groups, respectively), or were subcutaneously injected with the same volume of corn oil (control group). The doses of EB used in the present experiment were set in accordance with a report showing effects of EB on the number of apoptotic cells in sexually dimorphic nuclei of rats [2]. The EB25 and EB50 groups, the control group, and male pups were maintained with maternal care until MPNc tissue samples were obtained from them. On PD6 (18–21 h after EB treatment), in order to obtain the MPNc tissue samples, pups were decapitated, and then the brain was removed. Brain slices at the chiasmatic level were cut at a thickness of 400 μm using a microslicer (Dosaka EM, Kyoto, Japan). Immediately after the brain slices were obtained, tissue fragments of the MPNc were bilaterally isolated using a stainless steel pipe (inner diameter, 0.65 mm) and quickly frozen. The frozen tissues were kept at −80 °C until they were used for Western blotting for Bcl-2 and Bax. In addition, a part of the caudate putamen (CP) was isolated from 400-μm-thick brain slices using a stainless steel pipe (inner diameter, 0.9 mm), and the protein levels of Bcl-2 and Bax in the CP tissue were also examined.

After the MPNc and CP tissues were isolated, the brain slices were fixed with 10% formalin–saline, and stained with NeuroTrace 500/525 green fluorescent Nissl stain solution (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's protocol. The stained slices were observed under a fluorescent microscope to determine the location of the removed tissue.

Isolated tissue fragments were homogenized in lysis buffer (CelLytic; Sigma Chemical Co., St. Louis, MO) containing a protease inhibitor cocktail (1:4000; Sigma). The homogenate was centrifuged at 16,000 × *g* for 30 min at 4 °C. The protein concentration of the supernatant was determined using a QuantiPro BCA assay kit (Sigma), and the supernatant was then mixed with 1/4 volume of 0.29 M Tris–HCl (pH 6.8) containing 8.3% sodium dodecyl sulfate, 25% glycerol, 7.75% dithiothreitol, and 0.01% bromphenol blue. After the samples were heated at 95 °C for 4 min, equal amounts of protein were separated by electrophoresis on 12.5% sodium dodecyl sulfate-polyacrylamide gels ($n = 6$ for control females; $n = 4$ for EB25 and EB50 females; $n = 5$ for males). The proteins were electrophoretically transferred from the gel to a polyvinylidene difluoride membrane.

After transfer, the membranes were rinsed in TBST (20 mM Tris–HCl (pH 7.6), 0.9% NaCl, and 0.1% Tween-20) and blocked with 5% nonfat dry milk in TBST for 1 h at room temperature. After rinsing in TBST, the membranes were incubated overnight at 4 °C in the blocking buffer containing mouse anti-Bcl-2 antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-Bax antibody (1:1000; Cell Signaling Technology, Beverly, MA). Membranes were rinsed in TBST, and then were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000; Chemicon International, Temecula, CA) or horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000; Cell Signaling) in TBST for 1 h at room temperature. After rinsing in TBST, immunoreactive signals were visualized by applying Western lightning chemiluminescence reagent plus (PerkinElmer, Boston, MA) and exposing the membrane to X-ray film. The membranes were then rinsed in TBST, incubated with restore Western blot stripping buffer (Pierce, Rockford, IL) for 30 min at 37 °C, and then subjected to the above-described blotting process but using mouse anti-beta-tubulin antibody (1:2000; NeoMarkers, Fremont, CA) as the primary antibody.

Densitometry of immunoreactive signals was performed using ImageJ 1.31 (National Institutes of Health, Bethesda, MD). The density of signals for Bcl-2 or Bax protein was normalized by dividing it by the density for the beta-tubulin-immunoreactive signal in the same sample, and was expressed as the relative amount of protein (%), with the mean density of the control group set at 100%. Differences among groups in the protein levels of Bcl-2 and Bax were analyzed by one-way analysis of variance. When significant overall effects were detected, Fisher's PLSD test was performed as post hoc analysis. In the post hoc analysis, a probability value of $p < 0.05$ was considered to indicate statistical significance.

In fluorescent microscopy of brain slices after isolation of medial preoptic tissues, it was observed that the MPNc and a part of the medial preoptic nucleus surrounding the MPNc was bilaterally isolated from brain slices at the chiasmatic level (Fig. 1A and B). It was confirmed that all tissue fragments used for experiments contained the MPNc. Tissues of the CP were isolated from brain slices, as shown in Fig. 1C and D.

One-way analysis of variance indicated that there were significant differences in the relative level of Bcl-2 protein (~26 kDa), which was normalized to the level of beta-tubulin (~55 kDa), in the MPNc among groups [$F(3,15) = 8.21$, $p < 0.005$]. The Bcl-2 protein level in the MPNc of EB50 females was significantly higher than that of the control females or EB25 females (Fig. 2A). The Bcl-2 protein level of EB25 females did not significantly differ from that of the control females. There was no significant difference in the Bcl-2 protein level between the EB50 females and normal males, although the control and EB25