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Inhibition of the norepinephrine transporter function in cultured bovine adrenal medullary cells by bisphenol A

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Abstract

We report here the effects of an environmental estrogen, bisphenol A, on norepinephrine (NE) transporter function in cultured bovine adrenal medullary cells. The effects of bisphenol A were compared to those of 17β-estradiol. Bisphenol A significantly inhibited [^{3}H]NE uptake by the cells in a concentration-dependent manner (1–100 μM). Kinetic analysis revealed that bisphenol A, as well as 17β-estradiol, noncompetitively inhibited [^{3}H]NE uptake. Bisphenol A and 17β-estradiol inhibited the specific binding of [^{3}H]desipramine to plasma membranes isolated from bovine adrenal medulla. As shown by Scatchard analysis of [^{3}H]desipramine binding, bisphenol A increased the dissociation constant (K_d) and decreased the maximal binding (B_{max}), indicating a mixed type of inhibition. 17β-Estradiol increased the K_d without altering the B_{max}, thereby indicating competitive inhibition. The present findings suggest that bisphenol A inhibits the function of the NE transporter by acting on a site different from that of 17β-estradiol in the adrenal medulla and probably in the brain noradrenergic neurons.

Keywords: Adrenal medullary cells; Bisphenol A; Environmental estrogens; Norepinephrine uptake; Norepinephrine transporter; 17β-Estradiol

1. Introduction

Natural estrogens (particularly 17β-estradiol) play an important role in differentiation, proliferation, homeostasis, and the female reproductive system through genomic mechanisms [1]. Estrogens also are reported to have an effect on a variety of cell functions in a nongenomic manner [2]. For instance, estrogens rapidly modulate neural functions of membrane proteins such as neurotransmitter receptors [3], transporters [4], and ion channels [5].

In addition to estrogens, environmental estrogens that are nonsteroidal substances with a diverse chemical structure mimic the actions of estrogens [6]. Environmental estrogens such as 4-octylphenol and *p*-nonylphenol have been reported to induce acute vascular relaxation by inhibiting L-type voltage-dependent Ca^{2+} channels in rat vascular smooth muscle [7]. Therefore, environmental estrogens might acutely influence the function of neurons through ion channels or transporters of cell membranes, as do estrogens.

The norepinephrine transporter (NET), located on the presynaptic membranes of noradrenergic nerve terminals, mediates the termination of neurotransmission by the reuptake of NE released into the extracellular milieu [8,9]. NET is also known to be a target for the tricyclic antidepressant desipramine and psychostimulants such as cocaine and amphetamine [10–12]. Human NET was the first NET

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Abbreviations: B_{max}, maximal binding; K_d, dissociation constant; K_m, Michaelis–Menten constant; KRH, Krebs–Ringer–HEPES; MEM, minimum essential medium; NE, norepinephrine; NET, norepinephrine transporter; and V_{max}, maximal velocity.

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cloned, and its mRNA was localized in the brainstem and adrenal medulla [13]. Adrenal medullary cells derived from the neural crest share many physiological and pharmacological properties with postganglionic sympathetic neurons and abundantly express the NET [14,15]. Lingen *et al.* [16] reported the functional and pharmacological characterization of the NET cloned from the bovine adrenal medulla. Although the distribution of adrenal NET may be different from that of noradrenergic neuron's NET [17], the pharmacological properties of the NET in adrenal medullary cells are quite similar to those of the NET in the central and peripheral noradrenergic neurons [18]. Furthermore, numerous regulations of transporter function and surface expression in peripheral and central noradrenergic neurons were similarly recognized in the adrenal NET. For example, activation of protein kinase C down-regulated the NET in SK-N-SH cells [19] and adrenal medullary cells [20]. Therefore, the adrenal medullary cells expressing NET provide a convenient model system to study the effects of intravenous anesthetics [21] or antipsychotic drugs [22] on NET function.

Bisphenol A, one of the environmental estrogens, is a monomer found in polycarbonate plastics and a constituent of epoxy and polystyrene resins employed extensively in the food-packing industry and in dentistry [23,24]. A previous report showed that bisphenol A released from polycarbonate flasks during autoclaving has estrogenic properties [6]. In addition, exposure to environmentally relevant doses of bisphenol A has been shown to advance puberty and alter the postnatal growth rate in mice [25].

In the present study, we examined the acute effects of bisphenol A on NET function in cultured bovine adrenal medullary cells, and compared the effects of bisphenol A with those of 17β-estradiol.

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: Eagle's MEM, Nissui Pharmaceutical; calf serum, L-norepinephrine, pargyline hydrochloride, L-ascorbic acid, bisphenol A, *p*-nonylphenol, and 17β-estradiol, Nacalai Tesque; collagenase, Nitta Zerachin; desipramine hydrochloride, Sigma; nisoxetine hydrochloride, Research Biochemicals International; L-[7,8-^{3}H]norepinephrine (34.0 Ci/mmol), Amersham International; and [benzene ring, 10,11-^{3}H]desipramine hydrochloride (73.0 Ci/mmol), New England Nuclear.

2.2. Isolation of bovine adrenal medullary cells and primary culture

Bovine adrenal medullary cells were isolated by collagenase digestion of the adrenal medulla, as described previously [26]. Cells were suspended in Eagle's MEM containing 10% calf serum, 60 μg/mL of aminobenzylpenicillin, 100 μg/mL of streptomycin, 0.3 μg/mL of amphotericin B, and 3.0 μM cytosine arabinoside and were maintained in a monolayer culture at a density of 4×10^6 cells/dish in 5% CO_2/95% air [27]. Experiments were conducted on cells that had been cultured from 2 to 5 days.

2.3. [^{3}H]NE uptake by cells

Cells (4×10^6/dish) were preincubated at 37° for various periods in KRH buffer (composition: 156 mM NaCl, 5.7 mM KCl, 1.1 mM $MgCl_2$, 2.2 mM $CaCl_2$, 10 mM HEPES, pH 7.4, and 10 mM glucose) in the presence or absence of the test compounds. The cells were further incubated with KRH buffer containing 10 μM pargyline, 100 μM ascorbic acid, and [^{3}H]NE (0.5 μM, 0.1 μCi) at 37° for 10 min in the presence or absence of the test compounds. To determine the saturation kinetics of [^{3}H]NE uptake, cells were incubated with various concentrations (1–30 μM) of [^{3}H]NE in the presence or absence of 30 μM 17β-estradiol and bisphenol A. After incubation, the cells were rapidly washed four times with l mL of ice-cold KRH buffer and solubilized in 1 mL of 10% Triton X-100. The radioactivity in the solubilized cells was counted in a liquid scintillation counter (LSC-3500E; Aloka). Nonspecific uptake was determined in the presence of 10 μM desipramine, and specific uptake was obtained by subtracting the nonspecific uptake from the total uptake.

2.4. [^{3}H]Desipramine binding to plasma membranes

Plasma membranes of bovine adrenal medulla were prepared as described previously [15]. The equilibrium binding of [^{3}H]desipramine to bovine adrenal medullary cell membranes was assayed as previously described [20]. The plasma membrane of the adrenal medulla was incubated with various concentrations of [^{3}H]desipramine in a binding buffer (composition: 135 mM NaCl, 10 mM Tris–HCl, pH 7.4, 5 mM KCl, and 1 mM $MgSO_4$) at 25° for 30 min. [^{3}H]Desipramine bound to the membrane was separated from free ligand by filtration through a GF/C glass fiber filter (Whatman), and the filter was washed three times with the ice-cold binding buffer. Specific binding of [^{3}H]desipramine was defined as total binding minus nonspecific binding, which was determined in the presence of 10 μM nisoxetine.

2.5. Statistics

Results are expressed as means ± SD of multiple determinations. Statistical analysis was carried out by one-way analysis of variance with post hoc mean comparison using Fisher's PLSD. When $P < 0.05$, differences were considered statistically significant.

3. Results and discussion

3.1. Effects of environmental estrogens and 17β-estradiol on [^{3}H]NE uptake by bovine adrenal medullary cells

We examined the effects of environmental estrogens and 17β-estradiol on [^{3}H]NE uptake by bovine adrenal medullary cells. The cells were pretreated with or without bisphenol A and 17β-estradiol at 30 μM for the indicated periods (0–30 min), and then were incubated for another 10 min with or without the test compounds (30 μM). Bisphenol A and 17β-estradiol caused a rapid decrease in [^{3}H]NE uptake during the first 10 min and subsequently a gradual decrease at 10–30 min (Fig. 1A). Pretreatment of cells with bisphenol A and 17β-estradiol for 10 min caused a significant inhibition of [^{3}H]NE uptake at 1 μM and produced a concentration-dependent inhibition with half-maximal inhibitory concentration (IC_{50}) values of 24.7 and 6.2 μM, respectively (Fig. 1B). Treatment of cells with bisphenol A and 17β-estradiol at 100 μM decreased [^{3}H]NE uptake by the cells to 15 and 24% of control, respectively (Fig. 1B). Treatment with 100 μM *p*-nonylphenol, another environmental estrogen, also significantly decreased [^{3}H]NE uptake to 76% of control. This inhibitory effect on [^{3}H]NE uptake was smaller than that of bisphenol A. The concentrations of bisphenol A used in the present study were comparable with *p*-nonylphenol concentrations that induce acute coronary vascular relaxation in the isolated, perfused rat heart [7]. In COS-7 cells transfected with bovine NET cDNA, treatment with bisphenol A inhibited [^{3}H]NE uptake in a concentration-dependent manner (data not shown). These findings suggest that the functions of both native and recombinant NET are modulated by the same mechanism of bisphenol A. To our knowledge, this is the first report revealing evidence for the inhibition of NET function by environmental estrogens. The mechanism by which environmental estrogens and 17β-estradiol inhibited [^{3}H]NE uptake was not clear. The potencies of environmental estrogens and 17β-estradiol on [^{3}H]NE uptake inhibition seem to be much lower than those for activation of nuclear estrogen receptors [28]. Furthermore, tamoxifen, an antagonist for nuclear estrogen receptors, did not abolish but rather enhanced the inhibitory effect of bisphenol A and 17β-estradiol on [^{3}H]NE uptake. However, by itself, tamoxifen inhibited [^{3}H]NE uptake at concentrations similar to those of 17β-estradiol (data not shown). These findings suggest that bisphenol A and 17β-estradiol suppress the NET function through an acute and nongenomic pathway but not via nuclear estrogen receptors.

Incubation of the cells with increasing concentrations of [^{3}H]NE (1–30 μM) showed that [^{3}H]NE uptake was a saturable process (Fig. 2A). From the Eadie–Hofstee analysis (Fig. 2B), we determined the Michaelis–Menten constant (K_m) value for [^{3}H]NE uptake to be 2.83 ± 0.35 μM, and the maximal velocity (V_{max}) to be 8.09 ± 0.46 pmol/4 × 10^6 cells/min in control cells. Bisphenol A and 17β-estradiol at 30 μM produced a significant reduction in V_{max} (4.79 ± 0.74 and 3.75 ± 0.25 pmol/4 × 10^6 cells/min) without altering the K_m (3.04 ± 0.16 and 3.11 ± 0.14 μM), indicating noncompetitive inhibition. This inhibitory mechanism of bisphenol A and 17β-estradiol on [^{3}H]NE uptake seems to be distinct from that of desipramine, an antidepressant, and of cocaine, a psychostimulant, both of which have shown competitive inhibition of [^{3}H]NE uptake in adrenal medullary cells [21].

3.2. Effects of bisphenol A and 17β-estradiol on [^{3}H]desipramine binding to plasma membranes isolated from bovine adrenal medulla

As shown in Fig. 3A, the specific binding of [^{3}H]desipramine to plasma membranes was saturable. Scatchard analysis of control [^{3}H]desipramine binding showed a

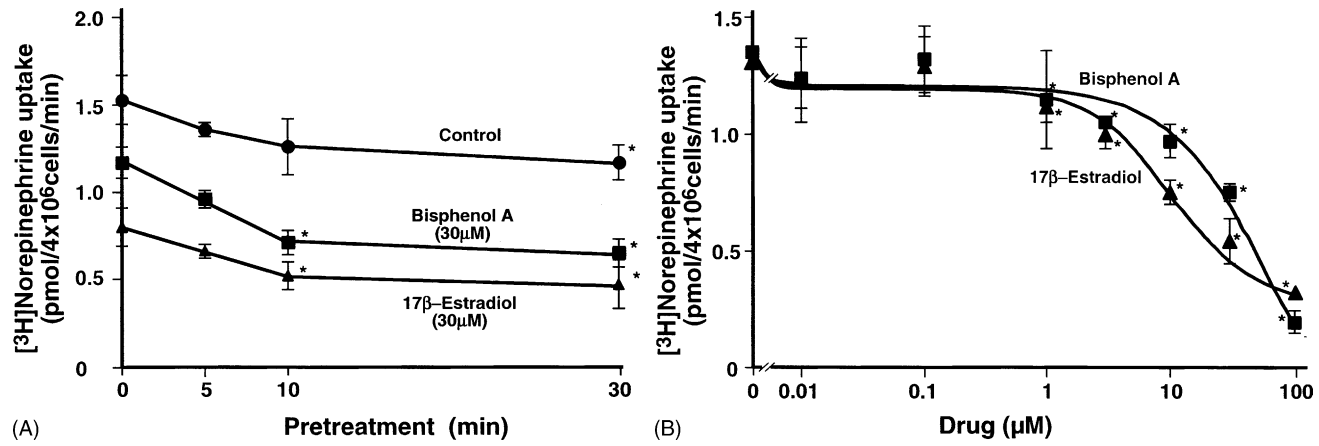

Fig. 1. Inhibition of [^{3}H]norepinephrine uptake by bisphenol A and 17β-estradiol in adrenal medullary cells. (A) Cells were pretreated with or without bisphenol A and 17β-estradiol (30 μM) for the indicated periods (0–30 min) before assessing [^{3}H]NE uptake. (B) Cells were treated for 10 min with various concentrations of bisphenol A or 17β-estradiol (10^{-8}–10^{-4} M), and then the desipramine-sensitive uptake of [^{3}H]NE by the cells was measured. Data are means ± SD from three separate experiments. Key: (*) $P < 0.05$, compared with each control.

	Km(μM)	Vmax (pmol/4x10^{6}cells/min)
Control	2.83 ± 0.35	8.09 ± 0.46
Bisphenol A	3.04 ± 0.16	4.79 ± 0.74*
17β–Estradiol	3.11 ± 0.14	3.75 ± 0.25*

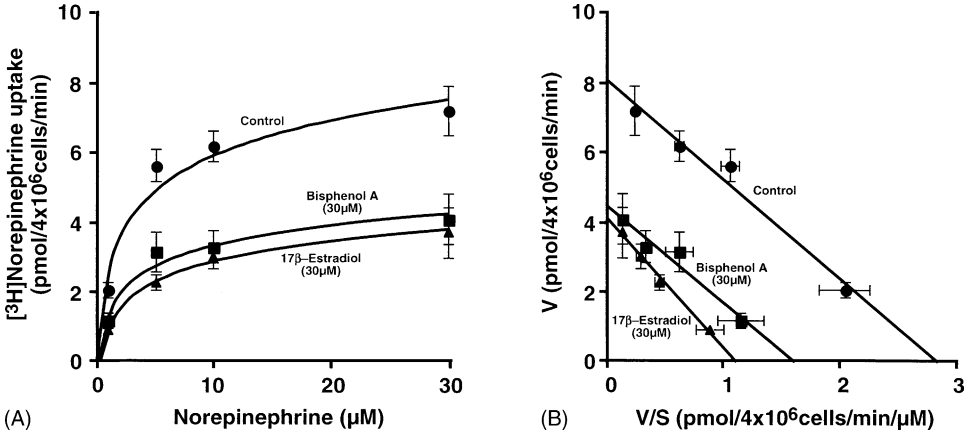

Fig. 2. Saturation curves and Eadie–Hofstee plots of [^{3}H]norepinephrine uptake. (A) Cells were treated with or without bisphenol A and 17β-estradiol (30 μM) for 10 min at 37°. Desipramine-sensitive [^{3}H]NE uptake was measured with various concentrations (1–30 μM) of [^{3}H]NE. (B) Eadie–Hofstee analysis of [^{3}H]NE uptake data shown in panel A. Data are means ± SD from three separate experiments. Upper table: K_m and V_{max} values were calculated by Eadie–Hofstee analysis of the saturation curves in the absence of a drug (control) or in the presence of 30 μM bisphenol A or 17β-estradiol. Key: (*) $P < 0.05$, compared with each control.

single population of binding sites with an apparent dissociation constant (K_d) of 3.89 ± 0.71 nM and maximal binding capacity (B_{max}) of 1.46 ± 0.33 pmol/mg of protein (Fig. 3B), in agreement with previous reports [15]. 17β-Estradiol (100 μM) inhibited [^{3}H]desipramine binding by increasing the K_d (10.02 ± 2.66 nM) without altering the B_{max} (1.51 ± 0.32 pmol/mg of protein), thus indicating competitive inhibition. On the other hand, bisphenol A (100 μM) increased the K_d (9.55 ± 0.64 nM) and decreased the B_{max} (0.72 ± 0.10 pmol/mg of protein), thereby suggesting a mixed type of inhibition. From these findings, we conclude that bisphenol A may interfere with [^{3}H]desipramine binding to the NET by acting on a site different from that of 17β-estradiol, which acts on a desipramine binding site.

Although the physiological relevance of our present findings remains to be demonstrated, it depends to a large extent upon the degree of exposure of wildlife and humans

	Kd(nM)	Bmax (pmol/mg protein)
Control	3.89 ± 0.71	1.46 ± 0.33
Bisphenol A	9.55 ± 0.64*	0.72 ± 0.10*
17β–Estradiol	10.02 ± 2.66*	1.51 ± 0.32

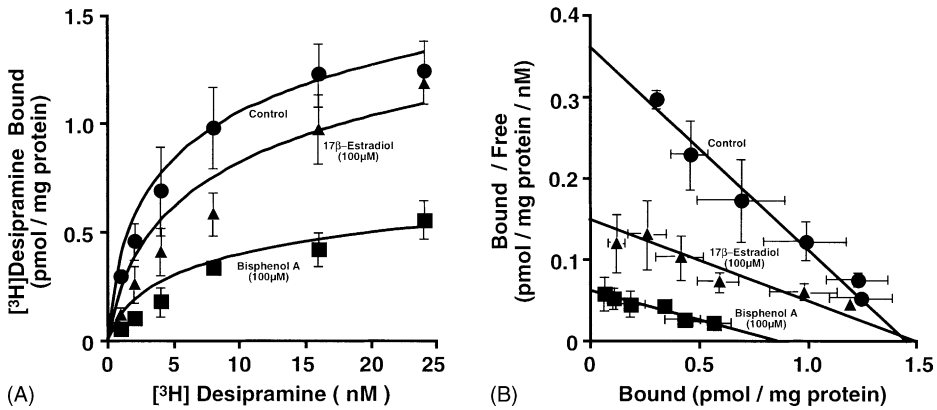

Fig. 3. Saturation curves and Scatchard plots of specific [^{3}H]desipramine binding to plasma membranes of bovine adrenal medullary cells. (A) Plasma membranes isolated from adrenal medulla (10 μg protein) were incubated at 25° for 30 min with or without bisphenol A and 17β-estradiol and in the presence of increasing concentrations of [^{3}H]desipramine (1–24 nM). Nonspecific binding was determined in the presence of 10 μM nisoxetine. Specific binding was 30–40% of the total binding at the K_d concentration of [^{3}H]desipramine. (B) The Scatchard plots are from one experiment that is representative of three separate experiments. Data are means ± SD from three separate experiments. Upper table: K_d and B_{max} values were calculated by Scatchard plot analysis in the absence of a drug (control) or in the presence of 100 μM bisphenol A or 17β-estradiol. Key: (*) $P < 0.05$, compared with each control.

to these environmental estrogens. Bisphenol A leaches into the human body from dental sealants and composite fillings and can be detected in saliva (up to 950 μg/hr) of patients with treated teeth [29]. Nagel *et al*. [30] reported that the free fraction of bisphenol A in human serum was 7.8%. Therefore, under physiological conditions, bisphenol A might not modulate the NET function since micromolar concentrations of bisphenol A are necessary to inhibit NE uptake. Because of its high lipid solubility, bisphenol A is distributed in various tissues including the lungs, kidneys, and testis, and even the brain in rats [31]. Sun *et al*. [32] reported that bisphenol A is capable of penetrating the blood–brain barrier of the rat. Pre- or postnatal exposure to bisphenol A has been reported to affect pain behavior induced by formalin injection in the rat [33], suggesting that perinatal exposure to bisphenol A permanently alters nociceptive responses in the central nervous system of the rat. Furthermore, Funabashi *et al*. [34] showed that subcutaneous injection of bisphenol A increases the expression of progesterone receptor mRNA in the preoptic area of adult ovariectomized rats. Therefore, it would be interesting to investigate whether exposure of perinatal or adult animals to environmental estrogens may affect the NET function of the brain. Presently, there is no direct evidence that the inhibitory effect of environmental estrogens on NET function is responsible for any deleterious effects on human health. However, we speculate that the widespread use or the persistence of environmental estrogens and their degradation products may pose a potential threat to human mental health, because the NET is known to be largely responsible for the efficient termination of noradrenergic neurotransmission in the brain, including the brainstem [13], which, in turn, plays an important role in emotional behaviors.

In conclusion, environmental estrogens inhibit NET function by acting on a site distinct from that of 17β-estradiol in the adrenal medulla. It is likely that noradrenergic neurons in the brain are therefore also modulated by these environmental estrogens.

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