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EFFECTS OF HINDBRAIN INFUSION OF AN ESTROGEN RECEPTOR ANTAGONIST ON ESTROGENIC MODULATION OF EATING BEHAVIOR

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Estradiol (E_2) inhibits eating behavior via activating estrogen receptors (ERs) within the brain. Activation of hindbrain ERαs has been shown to be sufficient to inhibit eating behavioral pattern. To investigate the involvement of hindbrain ERαs in estrogenic control of eating behavior, intracerebroventricular infusion (4th i.c.v.) of an estrogen receptor antagonist, ICI 182780 (ICI), was performed in ovariectmized female rats. Significantly lower daily food intake was observed in rats after estradiol benzoate (EB) injections. The effect of EB on food intake was significantly compromised by 4th i.c.v. infusions of both 4 and 8 nM ICI solutions. The results suggest that hindbrain infusions of ICI can significantly attenuate the inhibitory effect of E_2 on food intake. Importantly, 4th i.c.v. infusions during 12 days exerted no effect *per se* on eating. Further, there was no difference in the number of ERα immunopositive neurons in the selected hypothalamic nuclei and *nucl. tractus solitarius*. We conclude that the 4th i.c.v. infusions with ICI attenuated the exogenous estrogenic effect on food intake in ovariectomized rats, and the hindbrain is an important site providing estrogenic control of food intake.

KEYWORDS: ER antagonist, ICI 182780, estradiol, female rats, hindbrain, food intake.

INTRODUCTION

Several neuronal circuits have been shown to affect various aspects of eating behavior [1, 2]. The decreased eating behavioral pattern during a periovulatory phase in females is a distinct behavioral difference of such animals from males. This behavioral pattern is mediated mainly by estradiol (E_2). Unlike the effect of E_2 on copulation-related lordosis behavior [3], the neural circuit of E_2 affecting eating behavior has been only partially revealed. So, a specific brain site(s) by which E_2 affects eating behavior remains to be clarified. A number of the brain nuclei were shown to participate in the organization of this behavior [4]. Application of E_2 to the hypothalamic nuclei and its infusion directly into the hindbrain of experimental rats were shown to be sufficient to decrease the eating behavioral pattern [4, 5]. Knockdown of specific estrogen receptors α (ERαs) in the *nucl. tractus solitarius* (NTS) was demonstrated to attenuate the E_2 effect on eating [4, 6, 7]. Infusion of an estrogen receptor (ER) antagonist, ICI 182780 (ICI), could reverse the effect of E_2 on eating behavior [8]. Data on the potential involvement of the hindbrain in estrogenic inhibition of the eating behavioral pattern (although there are some evidences) are rather limited [4, 6, 7]. Thus, the aim of our study was to investigate whether changes in estrogenic inhibition of the eating behavioral pattern are mediated by ERα-positive neurons of the hindbrain.

METHODS

Animals. Female Wistar rats (National Laboratory Animal Care, the Mahidol University) weighing around 250–300 g were housed individually in hanging cages (33×18×20 cm) in a room maintained at 22 ± 2°C with a 12/12 light/dark cycle (light on 00 h). All rats had pelleted chow *ad libitum* and tap water. Rats were adapted to housing conditions for at least one week before the experiment started. Daily food intake (FI, ± 0.1 g corrected for spillage) was measured throughout the experimental period.

Ovariectomy. Female rats were ovariectomized

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in each experiment. Rats were anesthetized with isoflurane (2.5-3%, Minrad Inc., USA) and bilaterally ovariectomized using an intraabdominal approach. Immediately after surgery, rats were subcutaneously injected with enrofloxacin (2.5-5.0 mg/kg, i.v.; Bayer Korea Ltd., Korea) for antibiotic prophylaxis. Ibuprofen (Reckitt Benckiser Inc., Great Britain) was given once orally (15 mg/kg, p.o.) and, via drinking water (12 mg/100 ml), at 4 days to minimize post-surgical pain [5].

Fourth Ventricle Cannulation, Infusion, and Verification. Cannulation of the fourth ventricle was performed to provide possibilities for intracerebroventricular infusions (4th i.c.v.) of ICI to study the effect of exogenous E_2 on FI. Seven days after ovariectomy, rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p., Nembutal®, Ceva Santé Animale, France). A guide cannula (22 G, PlasticsOne, Roanoke, USA) was stereotaxically positioned into the fourth ventricle. The cannula tip was placed 3.5 mm posterior to the interaural line, 1.4 mm lateral to the midline, and 6.2 mm ventral to the skull surface [9]. The cannula was fixed to the skull with stainless steel mounting screws and dental cement. The cannula was attached to an osmotic pump (OP, Alzet Model 1002); the pump had a 100 µl reservoir volume. The rate and duration of infusions were 0.25 µl/h and 14 days, respectively. The pump was then placed at a subcutaneous space just behind the cannula. After surgery, rats received antibiotics and analgesics, as described previously.

At the end of experiment, all rats were euthanized by i.v. injection of a high dose of pentobarbital sodium (65 mg/kg). To verify the cannula placement in the second experiment, 5 µl of Evans Blue was slowly injected through the i.c.v. cannula. After the cannula was carefully removed, the brain was isolated, frozen, and sectioned to confirm the position of the cannula tip.

Continuous Infusion of ICI into the Fourth Ventricle and Estrogenic Control of Food Intake. The first experiment was performed to investigate the influence of hindbrain ERα blockage on the E_2-induced effect on FI. The 4th i.c.v. ICI infusions were performed over a period of 14 days. For each rat, the cannula was incerted directly into the 4th ventricle and connected with the OP containing either ICI solution (4 or 8 nM, n = 10 for each group) or vehicle (1% DMSO in normal saline). Rats were allowed five days to recover after surgical preparation. At postsurgery day 6, thirty rats were divided into two groups, 15 rats in each. Each rat in the first group was treated by single subcutaneous (s.c.) injection of 2 µg estradiol benzoate (EB, Sigma-Aldrich, USA), which was followed by the second single s.c. injection with sesame oil as a vehicle on postsurgery day 12. Each rat of the second group was treated by, first, a single s.c. injection with sesame oil as a vehicle, which was followed by the second single s.c. injection by EB on postsurgery day 12. Such EB injection, according to our previous data, could mimic the plasma estradiol effects on eating behavior in intact female rats [10]. With this treatment paradigm, the effects of EB on FI were analyzed two days after EB or vehicle injections into rats of both groups (postsurgery days 8 and 14).

Continuous Infusion of ICI into the Fourth Ventricle and Brain ERα Expression. If hindbrain ICI infusion could block or attenuate the E_2 effect on FI, the next experiment was designed to investigate whether 14-day-long ICI infusion into the hindbrain *per se* influenced FI and to identify the possible mechanism by which ICI attenuated the E_2 effect on FI. In this experiment, ovariectomized rats (n = = 10 per group) were divided into those obtaining 8 nM ICI or 1% DMSO 4th i.c.v. infusions, as was described above. Daily FI was measured without EB or oil injection until a day before the end of experiment. At postsurgery day 13, rats were deeply anesthetized and perfused. Brains were perfused and processed for ERα immunohistochemistry (ERα-IHC), as described previously [5]. Briefly, brain intranuclear ERαs were detected by rabbit polyclonal ERα antibody (c1355, 1:10,000; Upstate Biotechnology, USA). The antigen and antibody complex was amplified and strained using the avidin-biotin complex and DAB reaction (PK-6100 and SK-4105, Vector lab., USA), respectively. Numbers of ERα-positive neurons were counted within the following areas of interest (locations are millimeters caudal to the bregma) using templates based on the atlas of Paxinos and Watson [11]: NTS (the NTS subregions were based on our own nomenclature [5]); caudal NTS, cNTS, about 14.1-14.4 mm, and subpostremal NTS, spNTS, about 13.7-14.0 mm), medial preoptic nucleus (MPO; 0.4-0.92 mm posterior to the bregma), arcuate nucleus (Arc; 2.8-3.1 mm posterior to the bregma), and ventromedial nucleus of the hypothalamus (VMH; 2.3-2.8 mm posterior to the bregma). In the current experiment, one section from each nucleus of all animals was selected, based on the specific area of interest and a previous report that ERα neurons from the cNTS apparently influence the E2 effect on eating [5]. Cells were considered

ERα-positive ones if their nuclei contained punctate brown-black immunolabeling and were quantified blindly using constant minimum and maximum OD and object-size criteria, which were validated with visual counts.

Statistical Methods. Data from the experiment that contain either multiple time points or two factors were analyzed using two-way analysis of variance (ANOVA). Significant main effects were followed up using the Bonferroni post-test. Data of two experimental groups were compared with the Student *t*-test. All data are presented below as means ± s.e.m.

RESULTS

Continuous Infusion of ICI into the Fourth Ventricle and Estrogenic Control of Food Intake. Continuous administration of the ERα antagonist (ICI) into the hindbrain via the 4th ventricle cannulation revealed that both 4 and 8 nM ICI solutions could attenuate significantly the exogenous estradiol effect on FI (Fig. 1).There was a clear effect of estradiol on FI ($F_{1,26}$ = 15.99; $P < 0.05$). This EB effect on FI came mainly from the 1% DMSO hindbrain infusion group (t_{18} = 3.34, $P < 0.05$). However, daily FI values after 4 and 8 nM ICI hindbrain infusion did not differ significantly from those observed after oil and EB treatments (t_{18} = 2.04 and 1.49, $P > 0.05$). In addition,

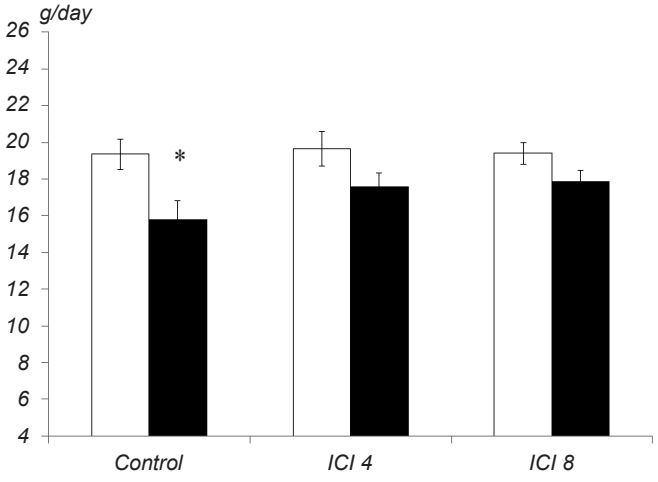


Fig. 1. Attenuation of the estrogenic inhibitory effect on food intake (FI) by hindbrain infusions of ICI. Mean values ± s.e.m. of daily FI, g/day, are shown. Open columns) FI with no manipulations; filled columns) FI affected by injections of estrogene benzoale with no infusion of ICI (control) and after infusion of 4 and 8 nM ICI solutions (ICI 4 and ICI 8, respectively).

Рис. 1. Послаблення гальмівного впливу естрогену на споживання їжі, обумовлене інфузіями ICI у задні відділи мозку.

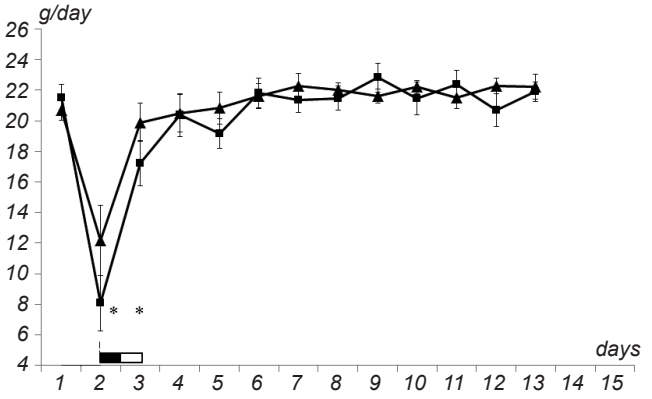


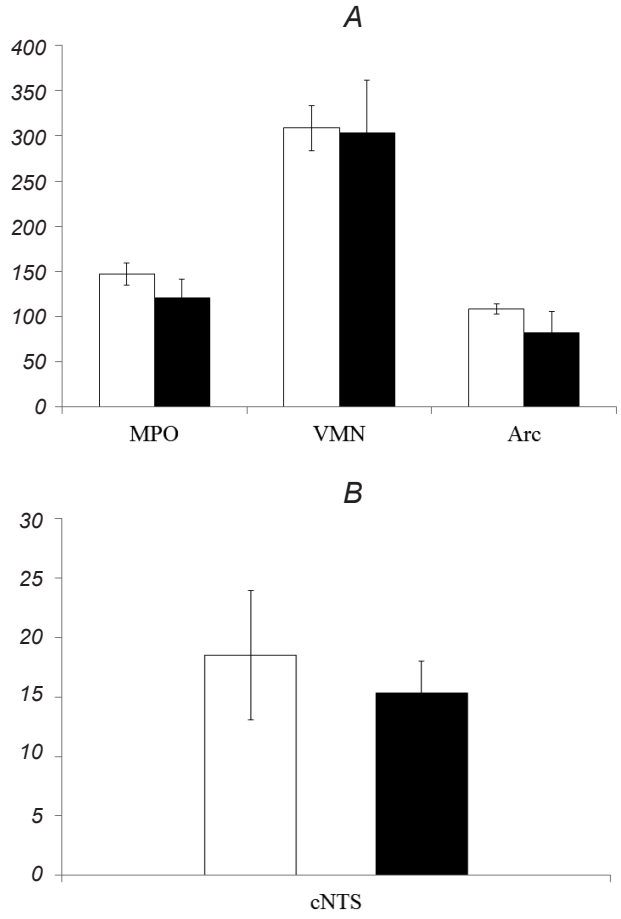
Fig. 2. Hindbrain infusion of ICI does not affect significantly daily FI (ordinate) across 12 days (abscissa). The dynamics of FI in rats infused with 1% DMSO (1) and ICI (8 nM, 2) do not differ significantly from each other. Drops in FI within initial two days are a result of surgery.

Рис. 2. Інфузії ICI у задні відділи мозку самі по собі не впливають на денне споживання їжі протягом 12 діб.

there were no significant difference between the effects of ICI among the treatment groups ($F_{2,26}$ = 0.99; $P > 0.05$).

Continuous Infusion of ICI into the Fourth Ventricle and Brain ERα Expression. This experiment aimed at elucidation of the effect of 4th i.c.v. of ICI on FI throughout the experimental period and of the potential mechanism by which ICI attenuated the E_2 effect on FI. First, hindbrain ICI infusion alone had no considerable effect on FI (Fig. 2). Daily FI in both 1% DMSO and 8 nM ICI hindbrain-infused groups did not significantly differ from each other throughout the experimental period ($F_{1,216}$ = 0.95; $P > 0.05$). However, this index decreased significantly under the action of surgical preparation ($F_{12,216}$ = 23.36; $P < 0.05$), but only immediately (about two days) after this intervention. Importantly, the levels of daily FI on day 3 after surgical preparation returned to the normal level identical to those before surgical preparation (t_9 = 0.15 and 0.86; $P < 0.05$).

Second, results from ERα IHC revealed that 8 nM ICI hindbrain infusion exerted no significant effect on the number of ERα-positive neurons in the selected hypothalamic nuclei (Fig. 3A), including MPO, VMH, and Arc (t_8 = 0.92, 0.09, and 1.10; $P > 0.05$, respectively). In addition, there was also no significant difference in the number of ERα-positive neurons in the cNTS (Fig. 3B, t_8 = 0.25; $P > 0.05$), while some trends toward decrease in ICI-infused animals were noteceable.



F i g. 3. Numbers of estrogen receptor α-positive (ERα) cells within the selected hypothalamic nuclei (A) and caudal *nucl. tractus solitarius* (B). MPO is the medial preoptic nucl., VMH is the ventromedial hypothalamic nucleus, and Arc is the *nucl. arcuatus*. Open columns) Values in estrogen-injected rats (4th i.c.v. control rets, 1 % DMSO); filled columns) values in rats that received additional infusions of ICI (8 nM).

Р и с. 3. Кількість клітин, позитивних щодо естрогенних рецепторів, в деяких гіпоталамічних ядрах (*А*) та *nucl. tractus solitarius* (*Б*).

DISCUSSION

It is generally known that estrogens inhibit the eating behavioral pattern in female animals by activating ERs in the brain. Both the hypothalamic forebrain nuclei and hindbrain NTS appear to be the sites mediating this phenomenon. Several previous reports indicated that E_2 acts centrally in the brain, which provides decreased eating in female rats [1, 2]. Administration of small E_2 doses and ablation of the hypothalamic nuclei revealed the potential site(s) of the E_2 effect on eating behavior. However, the outcomes might be either positive or negative because of the differences in techniques and doses [12-20]. In addition, it is unlikely that only hypothalamic nuclei are involved in the estrogenic effect on eating. A number of studies have suggested that the effect of E_2 on cholecystokinin (CCK) and feeding-induced c-Fos may be mediated via the hindbrain [21–23]. Our previous studies [5] showed directly that activation of ERαs at the caudal NTS decreased the FI level, and the effect was partly associated with CCK satiation. Further studies using an ERα-specific siRNA knockdown technique also demonstrated that deletion of ERαs from the caudal NTS eliminated the estrogenic effect on eating, and that this was again related to CCK satiation [4, 6, 7]. Therefore, these findings indicate that the hindbrain NTS is one of the most important sites participating in this phenomenon. In our experiments, ICI infusion into the hindbrain could attenuate, but not block completely, the effect of E_2 on eating behavior. This result agrees with the data of previous experiments revealing the necessity of brain ERs for the effect of E_2 on EI. In these experiments, infusion of ICI directly into the lateral cerebral ventricle could attenuate the E_2 effect on FI as well [8]. Furthermore, with the same experimental paradigm it was found that hindbrain ICI infusion *per se* exerted no considerable effect on eating across 12 days. This result also rules out the possibility that ICI acts on eating behavior as a selective estrogen receptor modulator (SERM) [24-26], and that the ICI-ER complex at the hindbrain is not crucially important for eating behavior in female rats. Moreover, ER degradation induced by the ICI-ER complex *in vivo* might not be a significant mechanism for this behavior (see below). Taken together, it can be concluded that the 4th i.c.v. ICI could attenuate the E_2 effect on eating behavior, but that ICI hindbrain infusion *per se* exerts no effect on eating in ovariectomized female rats. Importantly, our experiments do not disclose whether hindbrain ERs are required for the E_2 effect on FI.

Antiestrogenic actions of ICI are different from those observed in other SERM groups [27]. Besides AF1 and AF2 ER transactivation blocking and dimerization-related impairment, estrogenic actions are inhibited most importantly by ICI, and this promotes ER degradation [28–31]. Fluctuations of the number of ER-immunopositive neurons in the brain have been demonstrated across the ovarian cycle in female rats [32-35]. If ER degradation is an important mechanism of the antiestrogenic effect of ICI, investigation of the effect of ICI hindbrain infusions on the number of ER-immunopositive neurons *in vivo* under our experimental conditions

may reveal the mechanism of ICI action on the results observed. As was expected, the numbers of ERα-immunopositive neurons in the forebrain hypothalamic nuclei (MPO, VMH, and Arc) of control and ICI-infused rats were comparable. Likewise, the densities of ERα-immunopositive neurons in the cNTS were also nearly similar. Such a result suggests that infusion of ICI directly into the hindbrain does not affect significantly the number of ERs in the selected hypothalamic nuclei and NTS. Therefore, based on the ERα-immohistochemisty technique, the ICI-ER complex inducing ERα degradation *in vivo* is not apparently a crucial downstream mechanism for our results. Another possible *in vivo*-acting antagonistic mechanism of ICI was demonstrated by the measurements of [^{3}H]-labeled E_2 in the brain and uterus after ICI injections (experiments on Syrian hamsters and rats). The respective results clearly demonstrated that ICI suppressed cell nuclear binding of [^{3}H]-labeled E_2 up to 90% in the rat and to 50% in the Syrian hamster [36, 37]. It would be of interest to know whether ICI hindbrain infusion decreases the E_2-ER binding capability in the cNTS but not in the hypothalamic nuclei.

Thus, we can conclude that the above-described results made some contribution to identification of the brain site(s) where E_2 decreases the eating intensity in female rats. The attenuation effect of hindbrain ICI infusions on the estrogenic effect on eating suggests that such eating inhibitory effect of E_2 partly depends on activation of ERαs in the hindbrain. Together with our previous results [4, 6, 7], we conclude here that both siRNA ERα knockdown and ERα pharmacological antagonizing at the hindbrain (cNTS) could reverse the estrogenic effect on the eating behavioral pattern. The technique used can be applied as a pharmacological probe with respect to ERα agonists [38] and for investigations of the molecular actions of E_2 on eating behavior in female animals.

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All procedures were performed according to the international ethical principles and guidelines for the use of animals for scientific purposes from the National Research Council of Thailand and were approved by the Animal Use Committee (Faculty of Veterinary Science of the Chulalongkorn University).

The authors of this study, S. Thammacharoen, P. Kitchanukitwattana, P. Suwanapaporn, and N. Chaiyabutr, confirm that the research and publication of the results were not associated with any conflicts regarding commercial or financial relations, relations with organizations and/or individuals who may have been related to the study, and interrelations of co-authors of the article.

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ВПЛИВ ІНФУЗІЇ АНТАГОНІСТА ЕСТРОГЕНОВИХ РЕЦЕПТОРІВ У ЗАДНІЙ МОЗОК НА ЕСТРОГЕНІНДУКОВАНУ МОДУЛЯЦІЮ ХАРЧОВОЇ ПОВЕДІНКИ

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Р е з ю м е

Естрадіол (E_2) пригнічує харчову поведінку, і це опосередковується активацією церебральних рецепторів естрогенів (ER). Було показано, що для гальмування патерну харчової поведінки достатньо активації ER у задньому мозку. Щоб дослідити, чи необхідна активація цих рецепторів у даній частині мозку для естрогенопосередкованого контролю харчової поведінки, ми використовували інтрацеребровентрикулярні інфузії (4 i.c.v.) антагоніста естрогенових рецепторів ICI 182780 (ICI) оваріоектомованим самицям щурів. У таких самиць спостерігався вірогідно менший щоденний рівень споживання їжі після ін'єкції інфузій естрадіолу бензоату (EB). Вплив EB на споживанні їжі послаблювався після 4 i.c.v.-інфузій розчинів ICI в концентраціях як 4, так і 8 нМ. Наведені результати свідчать про те, що інфузії ICI в задній мозок можуть істотно нейтралізувати гальмівний ефект E_2. Важливо відмітити, що 4 i.c.v.-інфузії ICI протягом 12 діб самі по собі не впливали на харчування. Крім того, не було виявлено вірогідних відмінностей у кількості ERα-імунопозитивних нейронів у декількох гіпоталамічних ядрах та *nucl. tractus solitarius*. Отримані дані підтверджують, що 4 i.c.v.-інфузії ICI послаблюють вплив екзогенного естрогену на споживання їжі оваріоектомованими самицями щурів, а задні відділи мозку є важливим регіоном, що забезпечує естрогенопосередкований контроль споживання їжі.

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