The endogenous feeding suppressant, 2-buten-4-olide, impairs the pulsatile secretion of luteinizing hormone through endogenous opioid peptides

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Abstract

Objective: To clarify the mechanism of the suppressive effect of 2-buten-4-olide (2-B4O), an endogenous feeding suppressant, on the pulsatile secretion of luteinizing hormone (LH), by studying whether endogenous opioid peptides are involved in this suppressive effect.

Methods: Using ovariectomized (ovx) rats, blood samples were taken every 6 min for 2 h after administration of 2-B4O or saline into the third cerebroventricle (3V) and sequential i.v. injection of naloxone (0.5 mg/kg per h) or saline. Rats were divided into three experimental groups: group 1: 3V saline + i.v. saline (control); group 2: 3V 2-B4O + i.v. saline; group 3: 3V 2-B4O + i.v. naloxone. Serum LH concentrations were determined by double-antibody RIA. To determine whether 2-B4O affected the biosynthetic activity of the opioidergic neurons within the ovx rat arcuate nucleus, we measured the concentrations of pro-opiomelanocortin (POMC) mRNA, a precursor of β -endorphin, in the rostral arcuate nucleus using non-radioactive *in situ* hybridization and a computerized image-analysis system.

Results: 2-B4O significantly suppressed the pulse frequency of LH (group 2: 1.5 ± 0.33 pulses/2 h, group 1: 2.43 ± 0.2 pulses/2 h; $P < 0.05$), but naloxone blocked its suppressive effect and restored the pulse frequency (group 3: 3.29 ± 0.36 pulses/2 h, group 2: 1.5 ± 0.33 pulses/2 h; $P < 0.01$). There were no significant changes in the mean LH concentrations and amplitude. Furthermore, 2-B4O significantly stimulated the expression of POMC mRNA in the rostral arcuate nucleus.

Conclusion: These results suggest that 2-B4O may impair the pulsatile secretion of LH by activating the opioid pathway within the hypothalamus.

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Introduction

Reduced food intake is known to be associated with reproductive dysfunction in humans (1, 2), and rats (3–5). Several kinds of endogenous sugar acids found in rat blood have been reported to be involved in control of food intake (6, 7). It has been demonstrated that 2-buten-4-olide (2-B4O), which is one of the endogenous sugar acids and starts to increase 24 h after fasting in the blood of rats (8), induces a sensation of satiety and strongly suppresses feeding by i.p., i.v., third cerebroventricle (3V) and intragastric administration (9). It has also been suggested that the action of 2-B4O on food intake is mediated through glucose-sensitive neurons in the lateral hypothalamic area, a feeding center, and glucoreceptor neurons in the ventromedial hypothalamic nucleus of the hypothalamus, a satiety center (9).

Shitsukawa *et al.* (10) reported that administration of 2-B4O induced disturbance of the estrous cycle and a

decrease in the serum concentration of luteinizing hormone (LH) in intact rats. Saito *et al.* (11) also reported that administration of 2-B4O reduced the pulsatile secretion of LH from the pituitary in ovariectomized (ovx) rats. These results indicated that 2-B4O might impair the hypothalamic function, because the pulsatile secretion of LH is known to be maintained by gonadotropin-releasing hormone (GnRH) release. The mechanism of such an effect remains unclear.

There is general agreement that endogenous opioid peptides (EOPs) exert a suppressive effect on LH secretion. There are some reports (3–5) that fasting impairs the pulsatile secretion of LH and that the specific opioid antagonist, naloxone or naltrexone, reverses this impairment (12, 13). Therefore, EOPs may be involved in the 2-B4O-induced decrement in LH secretion.

In this study, to clarify the mechanism of impairment of the hypothalamic function by 2-B4O, we examined the hypothesis that 2-B4O may suppress the pulsatile secretion of LH through EOPs.

Materials and methods

Animals

Adult female Wistar–Imamichi rats (250–300 g) were purchased from Dohhan Ken (Saitama, Japan). They were housed in a room with controlled lighting (lights on between 0800 and 2030 h) and temperature (24 °C) and were given free access to standard laboratory pellets of rat chow and tap-water. All rats were ovariectomized (ovx) bilaterally under i.p. anesthesia with pentobarbital sodium (Nembutal, Abbot Laboratories, North Chicago, IL, USA; 40 mg/kg body weight) 2–4 weeks before each experiment.

Implantation of brain cannula

Three weeks after ovariectomy, a brain cannula was implanted under i.p. pentobarbital anesthesia (50 mg/kg body weight). A small hole was made in the skull with an electric drill. A guide cannula made of a 23-gauge stainless-steel tube (20 mm long, 0.64 mm o.d., 0.39 mm i.d.) was implanted into the third ventricle (3V) using the stereotaxic coordinates $AP = -1.5$ and $L = 0.0$ mm with respect to the bregma and $H = 7.5$ – 8.0 mm from the brain surface, according to the atlas of Paxinos & Watson (14). Two screws in the skull were used to anchor the cannula with dental cement. A sterile 29-gauge stainless-steel obturator with a polyethylene cap (18 mm long, 0.33 mm o.d.) was inserted into the guide cannula to ensure that the cannula remained patent. A new 29-gauge stainless-steel tube (18 mm long, 0.33 mm o.d., 0.17 mm i.d.) connected through a long polyethylene tube (0.6 mm o.d.) to a 10 μ l Hamilton microsyringe that was filled with freshly prepared test solution (2-B4O or saline), was inserted into the guide cannula. The length of the 29-gauge tube was adjusted to reach just to the tip of the guide cannula. Infusion was at a rate of about 1 μ l/10 s, to minimize the intraventricular pressure change. The infusion tube was long enough to permit manipulation of the syringe outside the cage, and to minimize restriction of the rats.

Atrial cannulation

On the day before the experiment, rats were anesthetized with a mixture of ketamine and xylazine (20:5 mg/kg i.p.) and a silastic tube (0.94 mm o.d., 0.51 mm i.d., Dow-Corning, Midland, MI, USA) was inserted into the external jugular vein and sewn into position in the right atrium (15). The tube was rinsed with heparinized saline (10 000 U/l saline) and threaded s.c. to an exit at the back of the neck. On the following day, the intra-atrial cannula was rinsed and connected to a long polyethylene tube containing heparinized saline. A steel pin was inserted into the open end of this tube, which was led outside the cage to permit rapid blood sampling without handling the rats.

Administration of 2-B4O

3V administration To examine the effect of naloxone on the impaired pulsatility of LH induced by 2-B4O, on days 7–10 after implantation of the brain cannula unrestrained ovx rats received a 3V administration of 5.0 μ mol/2 μ l 2-B4O (2(5H)-furanone, Aldrich Chemical Co., Milwaukee, WI, USA) or saline (2 μ l) only from 1100 h as previously described (11). Blood samples (0.3 ml) were taken every 6 min for 2 h through the intra-atrial cannula and an equal volume of heparinized saline containing naloxone (Sigma Chemical Co., St Louis, MO, USA) (0.5 mg/kg per h) or heparinized saline only was replaced after each bleeding (16, 17). Rats were divided into three experimental groups: group 1, 3V saline + i.v. saline (control); group 2, 3V 2-B4O + i.v. saline; group 3, 3V 2-B4O + i.v. naloxone. The samples were centrifuged and the plasma was stored at -40 °C until required for measurement of LH concentrations by RIA.

Atrial administration To examine the effect of 2-B4O on expression of pro-opiomelanocortin (POMC) mRNA in the arcuate nucleus, ovx rats (2 weeks after ovariectomy) were cannulated on the day before the experiment. On the experimental day 2-B4O (100 mg/kg body weight) dissolved in 0.5 ml saline or saline only was slowly injected through the intra-atrial catheter between 1330 and 1400 h. Animals were killed by decapitation 240 min after injection and the brains were rapidly removed from the skull, frozen on dry ice, and kept at -80 °C until sectioned. There were two rats in each treatment group.

RIA of LH

Serum LH was measured by double-antibody RIA (18) using a rat LH RIA kit obtained from NIDDK and the National Hormone and Pituitary Program. Values are expressed relative to those for the reference preparation (NIDDK-rLH-RP-3). The minimal detectable dose of this assay was 0.02 ng/tube and the intra- and interassay coefficients of variation (CV) were 8.6% and 9.2% respectively.

LH pulses were defined and identified exactly using established criteria as described by DePaolo *et al.* (19). Briefly, a CV was calculated from LH concentrations on the ascending and descending phase of a suspected pulse. A pulse was defined if the CV was greater than twice the CV of the LH RIA determined from solutions of LH standards corresponding to the mean LH levels of the suspected pulse. The mean LH pulse amplitude, pulse frequency (number of pulses per 2 h) and mean LH levels were calculated for each animal.

Probe preparation of POMC mRNA

A partial POMC cDNA clone of 423 bp, including the entire coding region for β -endorphin (20), was

subcloned in Bluescript KS(–). Digoxigenin-labeled sense and antisense RNA probes, corresponding to the POMC mRNA, were synthesized with a commercially available kit (DIG RNA labeling kit; Boehringer Mannheim GmbH, Germany).

In situ hybridization

Using the atlas of Paxinos & Watson (14) as an anatomical guide, 14 μ m coronal sections were cut throughout the rostral arcuate nucleus (bregma 2.3–2.5 mm) in a -15°C cryostat and thaw-mounted onto poly-L-lysine-coated slides. Frozen sections were fixed with 4% paraformaldehyde (pH 7.4) in PBS for 30 min, treated with proteinase-K (0.25 $\mu\text{g}/\text{ml}$ in 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) for 15 min, and fixed again in 4% paraformaldehyde for 10 min. After a wash with PBS, slides were treated with 0.2 M HCl for 5 min, and washed again with PBS. Slides were then rinsed in 0.1 M triethanolamine (pH 8.0), and acetylated with 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride for 10 min, washed with PBS, sequentially dehydrated in a series of graded ethanol (70%, 80%, 90%, 100%) and air dried.

After the application of 30 μl hybridization solution containing 50% formamide, 0.6 M NaCl, 1 $\times$ Denhardt's solution, 0.25% SDS, 10% dextran sulfate, 10 mM Tris–HCl (pH 7.6), 0.2 mg/ml yeast transfer RNA (tRNA) and 400 ng/ml DIG-11-UTP-labeled probe, slides were covered with coverslips and incubated in a moist chamber overnight at 50°C . After the removal of the coverslips, the slides were rinsed with 50% formamide– $2\times$ SSC for 30 min at 60°C and with TNE (10 mM Tris–HCl (pH 7.6), 0.5 M NaCl, 1 mM EDTA) for 30 min at 37°C . After RNase treatment (30 $\mu\text{g}/\text{ml}$ RNase-A in TNE) for 30 min at 37°C , the slides were rinsed in TNE for 10 min at 37°C , in $2\times$ SSC and twice in $0.2\times$ SSC for 20 min at 50°C .

The hybridization signal was detected using a Digoxigenin Detection Kit (Boehringer Mannheim GmbH) according to the manufacturer's instructions. The reaction was stopped by washing for 1 h in stop buffer (10 mM Tris–HCl (pH 7.6), 1 mM EDTA), and slides were mounted with Crystal/Mount (Biomedica Corp, Foster City, CA, USA).

We processed all 2-B4O-treated and control sections together, to ensure that all hybridization and color development conditions were standardized. To demonstrate the specificity of the signals, the following controls were performed: 1) adjacent tissue sections were hybridized with the POMC sense probe and 2) no probe was added to the hybridization solution.

Quantitative analysis of the amount of POMC mRNA

To determine the time of optimal color development of the hybridization signal in the rostral arcuate nucleus, a

time-course study was carried out. At predetermined times (15 and 30 min, 1, 2, 3, 5, 7 and 24 h), the color development was stopped. The number of tissue sections examined at each time was three. The color intensity of POMC mRNA-positive cells (more than 50 cells each time) in the rostral arcuate nucleus was measured using a computerized image-analysis system as described below.

Three photographs of the arcuate nucleus region ($\times 400$ magnification) in each tissue section were taken under bright-field illumination with a model BH-2 optical microscope (Olympus Optical, Tokyo, Japan) equipped with a camera (Olympus C-35AD-4, Tokyo, Japan), using positive film (Fujichrome Sensia Daylight 100, Fuji Film Corp, Japan). The films were digitized using a scanner (QuickScan 35, Minolta, Japan) with a Macintosh computer Quadra 800 and stored as an array of gray levels from 0 (white) to 256 (black). The stored signals were quantified with a computerized image-analysis program (NIH Image 1.59). The background gray level signal value was subtracted from the intensity of each POMC-positive cell. This corrected color intensity of each cell was averaged over replicates for each animal and used as an index of the relative cellular level of POMC mRNA.

Statistical analysis

The LH RIA data were analyzed with one-way analysis of test variance, followed by Fisher's protected least significance difference (PLSD) test. All results are presented as means $\pm$ S.E.M.

Statistical significance between results from 2-B4O-treated and control rats in the quantitative analysis of POMC mRNA was evaluated with Student's *t*-test, using $P < 0.05$ for the level of significance.

Results

Effect of naloxone on impaired pulsatility of LH by 2-B4O

Representative profiles of serum LH in ovx rats treated with 3V administration of 2-B4O or saline and sequential i.v. administration of naloxone or saline are depicted in Fig. 1. The mean pulse amplitude of LH, the pulse frequency of LH and the mean LH concentrations are shown in Fig. 2. There were no significant differences in the mean LH concentrations (group 1: $3.31 \pm 0.34 \mu\text{g}/\text{l}$, group 2: $2.94 \pm 0.34 \mu\text{g}/\text{l}$, group 3: $3.19 \pm 0.33 \mu\text{g}/\text{l}$; $P < 0.1$) and the mean LH amplitude (group 1: $4.13 \pm 0.31 \mu\text{g}/\text{l}$, group 2: $3.77 \pm 0.82 \mu\text{g}/\text{l}$, group 3: $2.70 \pm 0.52 \mu\text{g}/\text{l}$) among the three groups. However, the 3V administration of 2-B4O significantly suppressed the frequency of pulses (group 2: 1.5 ± 0.33 pulses/2 h, group 1: 2.43 ± 0.2 pulses/2 h; $P < 0.05$) and intermittent administration of naloxone significantly

Figure 1 Effect of third cerebroventricle (3V) administration of 2-buten-4-olide (2-B4O) and sequential i.v. administration of naloxone (NAL) on pulsatile release of LH in ovx rats: profiles of rats that received 3V injection of 2-B4O ($5.0 \mu\text{mol}/2 \mu\text{l}$) or saline ($2 \mu\text{l}$) at 0 min and sequential i.v. injections of naloxone (0.5 mg/kg per h) or saline every 6 min for 2 h. The rats were bled every 6 min for 2 h. Group 1: 3V saline + i.v. saline (control); group 2: 3V 2-B4O + i.v. saline; group 3: 3V 2-B4O + i.v. naloxone. Δ , pulse of LH secretion.

restored the frequency of pulses (group 3: 3.29 ± 0.36 pulses/2 h, group 2: 1.5 ± 0.33 pulses/2 h; $P < 0.01$). There was no significant difference in the frequency between groups 1 and 3.

Effect of 2-B4O on expression of POMC mRNA in the arcuate nucleus

Figure 3 depicts an example of the data obtained by *in situ* hybridization. Hybridization with the DIG-labeled antisense RNA probe yielded specific staining in the rostral arcuate nucleus (Fig. 3, top), whereas hybridization with the DIG-labeled sense RNA probe did not generate staining (Fig. 3, bottom). Specific staining was not observed without the probe being added to the hybridization solution (data not shown).

The relationship between the color intensity of the hybridization signal and color development time in rat rostral arcuate nucleus was investigated by a time-course study; the results are shown in Fig. 4. The intensity of the color signal concentrated within POMC

Figure 2 Effect of third cerebroventricle (3V) administration of 2-buten-4-olide (2-B4O) and sequential i.v. administration of naloxone on pulsatile release of LH in ovx rats. On days 7–10 after implantation of a brain cannula, at 1100 h, rats received a 3V injection of 2-B4O ($5.0 \mu\text{mol}/2 \mu\text{l}$) or saline ($2 \mu\text{l}$) at 0 min and sequential i.v. injections of naloxone (0.5 mg/kg per h) or saline every 6 min at bleeding time for 2 h. Group 1: 3V saline + i.v. saline (control); group 2: 3V 2-B4O + i.v. saline; group 3: 3V 2-B4O + i.v. naloxone. Values are expressed as means $\pm$ S.E.M. * $P < 0.05$ compared with group 1, ** $P < 0.01$ compared with group 3 (one-way analysis of test variance, followed by Fisher's protected least significance difference test).

mRNA-positive cells was found to increase up to 3 h and plateaued after that. It was important to ensure that all quantitative measurements were made while the curve was rising, therefore 30 min and 1, 2 and 3 h color development times were selected for the quantitative study.

Figure 5 depicts photomicrographs at each color development time that show the POMC mRNA positive-cells in the rostral arcuate nucleus of 2-B4O-treated and control rats. Analysis of POMC mRNA concentrations in individual cells of the rostral arcuate nucleus by a computerized image-analysis system revealed that 2-B4O-treated rats had a significant increase at all color development times compared with control rats (Fig. 6). There was no significant difference in the number of POMC positive cells between 2-B4O-treated and control rats.

Discussion

We have demonstrated that 2-B4O, which is an endogenous sugar acid and a feeding suppressant, impairs the pulsatile secretion of LH. We reported previously (10) that chronic i.p. treatment with 2-B4O disturbs the estrous cycle and diminishes the serum LH concentrations in intact rats. Moreover, we reported (11) that administration of 2-B4O via i.p., i.v. (100 mg/kg) and 3V ($5 \mu\text{mol}$) routes, in doses that decrease food intake, suppresses the pulsatile secretion of LH in ovx rats, and especially the pulse frequency. These results indicate that 2-B4O reduces LH pulsatile secretion. The pulsatile secretion of LH is known to be mediated by the GnRH pulse (21), therefore, 2-B4O may affect the hypothalamic function in ovx rats. However, its mechanism is unknown.

In our previous studies (10, 11), rats receiving chronic i.p. administration of 2-B4O had weight gains

Figure 3 Photomicrographs showing the rostral periaruate region ($\times 100$ magnification) after hybridization with a digoxigenin-labeled antisense probe (top) and a digoxigenin-labeled sense probe (bottom). 3V, third cerebroventricle. Scale bar represents $100\text{ }\mu\text{m}$.

similar to those of control rats. This finding suggests that 2-B4O may not have suppressed pulsatile secretion of LH through body weight loss. Next, we speculated that two factors might contribute to the impairment of GnRH secretion from the hypothalamus by 2-B4O: corticotropin releasing factor (CRF) and EOPs. We investigated CRF in a previous study (11), but found that pretreatment with 10 or $50\text{ }\mu\text{g}$ CRF antagonist did not block the suppressive effect of 2-B4O. This finding suggested that 2-B4O might not suppress the pulsatile

Figure 4 Relationship between the color intensity of the hybridization signal (gray level) and color development time in the rat rostral arcuate nucleus. Note that the intensity of the hybridization signal increases with color development time until approximately 3 h, after which it begins to plateau. Values are expressed as means $\pm$ S.E.M. More than 50 cells were sampled at each time.

secretion of LH through the CRF pathway. In the present study, we investigated whether EOPs were involved in the suppression of LH pulsatility.

There is general agreement that EOPs exert a suppressive effect on LH secretion. There are also several reports that fasting impairs the pulsatile secretion of LH (3–5) and that specific opioid antagonists reversed this impairment (12, 13). Therefore, EOPs might be involved in the 2-B4O-induced decrease in LH. The effect of 2-B4O on pulsatile secretion of LH cannot be analyzed clearly using adult female rats with ovaries because it may be affected by ovarian factors such as estradiol, progesterone, inhibin and activin. In our previous study (11), we found that the 3V administration of 2-B4O suppressed LH secretion most strongly. Therefore, we used ovx rats and the 3V route to administer 2-B4O.

The present results demonstrated that 3V administration of 2-B4O suppressed the pulse frequency of LH, as in our previous study, and intermittent administration of naloxone (0.5 mg/kg per h) after 3V administration of 2-B4O restored the pulse frequency. Funabashi *et al.* (16) reported that, in ovx rats, intermittent administration of naloxone in the same dose as we used resulted in a significant increase in the mean LH concentration. Kimura *et al.* (17) reported that the same intermittent administration of naloxone in ovx rats significantly increased the frequency and the duration of multiunit activity volleys that accompanied the initiation of each LH pulse. In the present study, naloxone did not increase the mean LH concentrations; therefore, the suppressive effect of 2-B4O on the pituitary response to GnRH might not be completely blocked by administration of naloxone. In contrast, naloxone restored the pulse frequency of LH secretion. This finding suggests that 2-B4O suppresses the GnRH release through EOPs.

β -Endorphin, which is an endogenous opioid peptide, is secreted by processing the precursor, POMC. The greatest concentration of POMC-derived peptides has been found in the arcuate nucleus (22). It has been suggested (23–26) that the POMC neurons from the arcuate nucleus to the medial preoptic area participate, either directly or indirectly via μ receptors, in the inhibition of GnRH neuronal activity. Bohler *et al.* (27), using radioactive *in situ* hybridization (ISH), reported that the pattern of changes in POMC mRNA concentrations in the rostral periaruate region was consistent with an inhibitory effect of β -endorphin on GnRH release. Therefore, we studied whether 2-B4O increased the biosynthesis of POMC mRNA within the rostral arcuate nucleus.

Larsen & Mau (28), using radioactive ISH and a computerized image-analysis system (NIH image ver 1.49), reported that, 240 min after initiation of the restraint stress, POMC mRNA concentrations within the arcuate nucleus were significantly increased. A computerized image-analysis system has also been reported (29) to be useful for quantitative non-radioactive ISH

Figure 5 Photomicrographs showing POMC mRNA-positive cells in rostral arcuate nucleus ($\times 400$ magnification) of 2-B4O-treated (left) and control rat (right) at 30 min and 1, 2 and 3 h.

with a digoxigenin-labeled probe. Therefore, to measure the POMC mRNA concentrations in the rostral arcuate nucleus 240 min after i.v. injection of 2-B4O (100 mg/kg), we used non-radioactive ISH with a digoxigenin-labeled probe and a computerized image-analysis system (NIH image 1.59/fat).

The present results demonstrate that the administration of 2-B4O also significantly increased the POMC mRNA concentrations within the rostral arcuate nucleus. Although the content and release of EOPs were not directly determined, this finding suggests that 2-B4O increases the biosynthesis of EOPs.

Figure 6 Color intensity of the hybridization signal (gray level) in the rostral arcuate nucleus of 2-B4O-treated and control rats at 30 min and 1, 2 and 3 h. Note that the intensity of the hybridization signal in POMC-mRNA positive cells was significantly increased in 2-B4O-treated rats at all times. ○, 2-B4O-treated rats; ■, saline treated-control rats. Values are expressed as means $\pm$ S.E.M. More than 50 cells were sampled at each time. * $P < 0.01$ compared with saline control, ** $P < 0.05$ compared with saline control (Student's *t*-test).

In conclusion, these results indicate that 2-B4O may impair GnRH release by activating the opioid pathway, and increases in 2-B4O and EOPs in the hypothalamus may contribute to starvation-induced reproductive dysfunction.

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