Effects of 2-buten-4-olide, an endogenous feeding suppressant, on the pulsatile secretion of luteinizing hormone in ovariectomized rats

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The effects of 2-buten-4-olide (2-B40), an endogenous feeding suppressant, on the secretion of luteinizing hormone (LH) were studied in ovariectomized rats. Intraperitoneal (ip) administration of 2-B40: adult female ovariectomized Wistar rats were given daily ip injections of solution containing 2-B40 at 0.50 or 100 mg/kg body wt for 14 days. This ip treatment with 2-B40 significantly decreased the mean LH concentration and pulse frequency of LH. Intravenous (iv) administration of 2-B40: a solution of 2-B40 (50 or 100 mg/kg body wt) was slowly injected through an intra-atrium catheter and blood samples were taken every 6 min for 2 h. This iv treatment significantly suppressed the LH pulse frequency but had no significant effect on the LH amplitude or mean LH. Injection of 2-B40 into the third cerebroventricle: the injection of 2-B40 into the third cerebroventricle of freely moving rats decreased the mean LH concentration and the frequency and amplitude of LH pulses. Third cerebroventricle injection of a corticotropin-releasing factor (CRF) receptor antagonist before third cerebroventricle injection of 2-B40: the specific CRF receptor antagonist α-helical-CRF (9–41) was injected into the third cerebroventricle of ovariectomized rats before injection of 2-B40. Administration of 2-B40 into the third cerebroventricle significantly decreased the mean LH concentration and pulse frequency. Third cerebroventricle injection of the CRF antagonist at 50 μg/rat and then 2-B40 also resulted in significant suppression of the mean LH concentration and pulse frequency. These findings suggest that 2-B40 may suppress gonadotropin-releasing hormone secretion in ovariectomized rats and that its effect in decreasing LH pulses may not be caused through the CRF pathway.

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Anorexia nervosa is a disorder of eating, associated with weight loss and amenorrhea, but its cause is not yet known. Recently, some kinds of organic acids found in the blood of rats were reported to be involved in control of food intake (1–3). One of them, 2-buten-4-olide (2-B40), strongly suppressed feeding via intraperitoneal (ip), intravenous (iv), third cerebroventricle (3V) or intragastric administration (4). It is suggested that 2-B40 induces the sensation of satiety through its effect on glucose-sensitive neurons in the lateral hypothalamic area and glucoreceptor neurons in the ventromedial nucleus of the hypothalamus (4). Reduced food intake is known to impair reproductive function in humans (5–7) and rats (8–12). Shitsukawa et al. reported that ip administration of 2-B40, which increases in the blood of starved rats, resulted in disturbance of the estrous cycle, decrease of the serum LH level and suppression of the pituitary response to GnRH in vitro (13). Therefore, 2-B40 may play an important role in starvation-induced anestrous.

On the other hand, CRF decreases LH secretion in humans (14) and rats (15, 16). The mean concentration of CRF in the cerebrospinal fluid are higher in patients with anorexia nervosa than in control subjects (17). The plasma corticosterone level was found to be increased by iv injection of 2-B40 and this increase was attenuated by anti-CRF antibody (18). Therefore, 2-B40-induced CRF may impair reproductive function in rats.

In this study, using ovariectomized (OVX) rats, we examined the effects of 2-B40 on the pulsatile secretion of LH to clarify the mechanism of its suppression of reproductive function.

Materials and methods

Animals

Adult female Wistar–Imamichi rats (250–300 g) were purchased from Doehan Ken (Saitama, Japan). They were housed in a room with controlled lighting (lights on between 08.00–20.30 h) and temperature (24°C) and were given free access to standard laboratory pellets of rat chow and tap-water. All the rats used in this study were ovariectomized bilaterally under anesthesia with pentobarbital sodium (Nembutal, Abbot Laboratories, North Chicago, IL; 40 mg/kg body wt ip) and used 2–3 weeks later.
**Atrial cannulation**

Rats were anesthetized with a mixture of ketamine and xylazine (20:5 mg/kg ip) and a silastic tube (0.94 mm o.d., 0.51 mm i.d., Dow-Corning, Midland, MI) was inserted into the external jugular vein and sewn into position in the right atrium (19). The tube was rinsed with heparinized saline (1 × 10^5 U/l saline) and threaded sc to an exit at the back of the neck. The next day, at 13.00 h, 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.

**Implantation of brain cannulae**

Ten to 14 days after OVX, brain cannulae were implanted under ip pentobarbital anesthesia (50 mg/kg body wt). A small hole was made in the skull with an electric drill. A guide cannula of 23-gauge stainless-steel tubing (20 mm long, 0.64 mm o.d., 0.39 mm i.d.) was implanted into the third ventricle 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 and Watson (20). 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.) flush with the tip of the guide cannula was used to ensure that the cannula remained patent (21).

**Intra-third cerebroventricle (3V) administration**

A 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-μl Hamilton microsyringe, all filled with freshly prepared test solution, 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. Infusions were performed at a rate of about 1 μl/10 s to minimize intraventricular pressure change. The infusion tubes were long enough to permit manipulation of the syringe outside the cage, and to minimize restriction of the rats (21).

**Experiment 1: effect of ip administration of 2-B4O on pulsatile LH release in OVX rats**

Adult female OVX Wistar rats were given daily ip injections of 2-B4O (2(5H)-furanone, Aldrich Chemical Co., Milwaukee, WI) at 50 or 100 mg/kg body wt in saline (1 ml) or saline only between 13.30 and 14.00 h. On day 13 of injections, their jugular veins were cannulated. From 15.00 h on day 14, blood samples (0.25 ml) were taken every 6 min for 2 h from these unanesthetized and unrestrained rats through the intra-atrial cannula. At each sampling time, the blood was replaced by an equal volume of heparinized saline. The samples were centrifuged and the plasma was stored at −40°C for subsequent determination of LH levels by RIA.

**Experiment 2: effect of iv administration of 2-B4O on pulsatile LH release in OVX rats**

Rats were anesthetized and a jugular vein was cannulated. At 15.00 h on the following day, a blood sample (0.25 ml) was withdrawn from these unanesthetized and unrestrained rats and designated as the 0-min sample. Then 2-B4O (50 or 100 mg/kg body wt) dissolved in 0.5 ml of saline, or saline only (0.5 ml), was slowly injected through the intra-atrium catheter. Blood samples were taken, centrifuged and stored as described above.

**Experiment 3: effect of 3V injection of 2-B4O on pulsatile LH release in OVX rats**

On day 10–14 after implantation of brain cannulae, a blood sample was taken and designated as the 0-min sample. Then each conscious, unrestrained animal received a 3V injection of 2-B4O (2.5 or 5.0 μmol/2 μl of saline) or saline only at 15.00 h. Blood samples were taken every 6 min for 2 h, centrifuged and stored as described above.

**Experiment 4: influence of 3V injection of CRF receptor antagonist on the effect of 2-B4O on pulsatile LH release in OVX rats**

At 14.00 h on day 10–14 after implantation of brain cannulae, a blood sample was taken and designated as the 0-min sample. Then the conscious, unrestrained animals received a 3V injection of the specific CRF receptor antagonist (α-helical CRF (9–41), Sigma Chemical Co., St Louis, MO) at 10 or 50 μg/4 μl of saline or saline only. After 1 h, the rats were treated 3V with 2-B4O at 5.0 μmol in saline or saline only. Blood samples were taken every 6 min for 2 h, centrifuged and stored as described above.

**Radioimmunoassay of LH**

Luteinizing hormone was measured by double-antibody RIA (22) using a rat LH RIA kit obtained from the NIDDK. Values are expressed relative to those for the reference preparation (NIDDK-rLH-RP-3). The sensitivity of the assay was 0.02 ng/tube and the intra- and interassay variabilities were 5.8% and 8.6%, respectively.

Luteinizing hormone pulses were defined and identified exactly using established criteria as described by DePaolo et al. (23). Briefly, a coefficient of variation (cv) was calculated from LH levels 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. Similar determinations and calculations were performed for each treatment group.

**Statistics**

All results are presented as means ± SEM. The data were analyzed with a one-way analysis of test variance, followed by the Bonferroni method (24). The Bonferroni critical significance level obtained from the tables of the t-distribution using a significance level of p/m, where m is the number of comparisons between groups to be performed. In this study, the p value of 0.05 would be replaced by 0.05/2 = 0.025.

**Results**

**Experiment 1: effect of ip administration of 2-B4O on pulsatile LH release in OVX rats**

Administration of 2-B4O ip significantly suppressed the mean LH concentration (control, 2.70 ± 0.23 μg/l vs 2-B4O (100 mg/kg), 1.72 ± 0.17 μg/l; p < 0.01), the LH pulse frequency (control, 3.00 ± 0.44 pulses/2 h vs 2-B4O (100 mg/kg), 1.22 ± 0.28 pulses/2 h; p < 0.01) (Fig. 1).

**Experiment 2: effect of iv administration of 2-B4O on pulsatile LH release in OVX rats**

Representative LH secretion profiles for the six individual animals treated iv with 2-B4O in saline or saline only are depicted in Fig. 2. Administration of 2-B4O iv promptly decreased the LH pulse frequency compared with that after saline treatment. The inhibition of LH release caused by injection of 2-B4O at 100 mg/kg was associated with the disappearance of detectable pulses of LH throughout the 2-h sampling period in several animals. Other animals receiving this dose of 2-B4O exhibited few, low-amplitude pulses during the 2-h sampling period.

The iv administration of 2-B4O significantly suppressed the LH pulse frequency (control, 2.00 ± 0.54 pulses/2 h vs 2-B4O (100 mg/kg), 0.43 ± 0.20 pulses/2 h; p < 0.02) but had no significant effect on the LH amplitude or mean LH level (Fig. 3).

**Experiment 3: effect of 3V injection of 2-B4O on pulsatile LH release in OVX rats**

Representative LH secretion profiles for the six individual animals treated 3V with 2-B4O or saline only are displayed in Fig. 4.

Injection of 2-B4O into the third cerebroventricle significantly decreased the mean LH concentration (control, 2.25 ± 0.24 μg/l vs 5.0 μmol 2-B4O,
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2.5umol2-B40
was dissolved in 0.5 ml of saline, or saline only (0.5 ml) was slowly injected through the intra-atrium catheter. Blood samples were taken every 6 min for 2 h. The histograms summarize the effects of iv administration of 2-B40 on the mean level, pulse amplitude and pulse frequency of LH secretion during 2-h sampling period (mean ± SEM). *p<0.025 (vs saline controls); **p<0.02 (vs saline controls).

Fig. 4. Experiment 3: effect of third cerebroventricle (3V) administration of 2-buten-4-olide (2-B40) on pulsatile LH release in ovariectomized (OVX) rats. The profiles of secretion are for individual OVX rats treated iv with 2-B40 (2.5 or 5.0 μmol/2 μl saline) or saline at 0 min. Δ denotes a pulse of LH secretion.

1.40±0.18 μg/l; p<0.01), the frequency of LH pulses (control, 2.63±0.33 pulses/2 h vs 5.0 μmol 2-B40, 1.14±0.34 pulses/2 h; p<0.01) and LH amplitude (control, 2.13±0.29 μg/l vs 5.0 μmol 2-B40, 0.94±0.18 μg/l; p<0.02) (Fig. 5).

Fig. 5. Experiment 3: effect of third cerebroventricle (3V) administration of 2-buten-4-olide (2-B40) on pulsatile LH release in ovariectomized (OVX) rats. On day 10–14 after implantation of brain cannulae a blood sample, designated as the 0-min sample, was taken and then each rat received a 3V injection of 2-B40 (2.5 or 5.0 μmol/2 μl saline) or saline only. Blood samples were taken every 6 min for 2 h. The histograms summarize the effect of 3V administration of 2-B40 on the mean level, pulse amplitude and pulse frequency of LH secretion during the 2-h sampling period (mean ± SEM). *p<0.02 (vs saline controls); **p<0.01 (vs saline controls).

Fig. 6. Experiment 4: effect of 3V injection of CRF antagonist and 2-buten-4-olide (2-B40) on pulsatile LH release in ovariectomized (OVX) rats. At 1400 h on day 10–14 after implantation of brain cannulae, a blood sample, designated as the 0 min sample, was taken. Then each rat received a 3V injection of the specific CRF receptor antagonist (α-helical-CRF [9–41]), (10 or 50 μg/4 μl saline) or saline only. After 1 h, the rats were treated 3V with 2-B40 at 5.0 μmol in saline or saline only. Blood samples were taken every 6 min for 2 h (means ± SEM). *, p<0.02 [vs saline controls]. **p<0.01 [vs saline controls].

Experiment 4: influence of 3V injection of CRF receptor antagonist on the effect of 2-B40 on pulsatile LH release in OVX rats
The mean LH secretion profiles in animals treated 3V with CRF antagonist or saline and then 2-B40 or saline are shown in Fig. 6. The 3V administration of vehicle (v) + 2-B40 significantly suppressed the mean LH level (v + v, 2.30±0.23 μg/l vs v + 2-B40, 1.57±0.20 μg/l; p<0.02) and the frequency of pulses (v + v, 2.67±0.23 pulses/2 h vs v + 2-B40, 1.00±0.29 pulses/2 h; p<0.01). The 3V injection of CRF antagonist at 10 μg (10 ant) or 50 μg (50 ant) + 2-B40 also significantly suppressed the mean LH level (10 ant + 2-B40,
Discussion

We have reported that ip treatment with 2-B4O disturbs the estrous cycle in rats (13). In this previous study, after 14 days of ip administration with 2-B4O at 100 mg/kg, rats with 2-B4O and control rats gained similar weight. Serum LH levels were low but the amount of LH in the pituitary gland increased on chronic ip treatment with 2-B4O. Moreover, the GnRH content of the mediobasal hypothalamus did not change and 2-B4O significantly suppressed GnRH-stimulated LH release from the pituitary gland in vitro. These data suggest that the suppressive effect of 2-B4O on LH secretion is mediated by suppression of the pituitary responsiveness to GnRH and not through body weight loss. However, we did not study pulsatile LH secretion. The effect of 2-B4O on LH pulsatile secretion cannot be analyzed clearly using mature female rats with ovaries, because it may be affected by ovarian factors such as estradiol, progesterone, inhibin and activin. Moreover, we administered 2-B4O only by the ip route although 2-B4O is effective in suppressing food intake via ip, iv or 3V (4). Therefore, it was necessary to administer 2-B4O by the ip, iv or 3V route by using OVX rats for the study of pulsatile secretion of LH.

In the present study, OVX rats were treated ip with the same dose as in the previous study (100 mg/kg) and we examined body weights of OVX rats. There were no significant differences in body weight among three groups (0, 50 and 100 mg/kg body wt. data not shown) or in the body weight of fertile rats in our previous study (13). We found that ip administration of 2-B4O suppressed the mean LH level in OVX rats. This result is consistent with the previous finding that the serum LH concentration was reduced in 2-B4O-induced anestrous rats. Moreover, we found that chronic ip administration of 2-B4O decreased LH pulse frequency. These results indicate that 2-B4O did not impair the pulsatile LH release through body weight loss.

In Experiment 2 and 3 we studied the other routes (i.e. iv and 3V) that effectively suppressed food intake (4). These studies clearly indicated that iv or 3V administration of 2-B4O, which sufficiently suppresses food intake, also impairs the LH pulse frequency. The pulsatile secretion of LH is known to be maintained by the GnRH pulse (25, 26). Thus, these findings suggest that 2-B4O also may affect hypothalamic function in OVX rats.

We confirmed that previous iv or 3V administration of 2-B4O (100 mg/kg, 5.0 μmol) significantly suppressed LH release from the pituitary after iv administration of 100 ng/kg body wt GnRH in OVX rats (data not shown). Therefore, in OVX rats, 2-B4O seems to inhibit both the hypothalamic and pituitary functions.

In this study, we used three routes of administration of 2-B4O: ip, iv and 3V. The suppressive effect of 2-B4O on LH pulse secretion was observed on its administration by each of the three routes. Treatment of rats with 2-B4O ip at a dose of 100 mg/kg or 3V at 5.0 μmol decreased their food intake (4). The doses of 2-B4O administered ip or 3V in this study also suppressed LH pulse secretion. These data suggest that a dose of 2-B4O that suppresses food intake also impairs reproductive function.

Corticotropin-releasing factor inhibits LH secretion in humans (14) and rats (15, 16), although the site of its action for attenuating LH secretion is controversial. The 3V administration of CRF is known to suppress food intake in rats (27–29). High CRF concentrations are present in the cerebrospinal fluid of patients with anorexia nervosa (17). The plasma corticosterone level was found to be increased by 2-B4O and this increase was attenuated by anti-CRF antibody application, but not by cutting the splanchic adrenal nerve (18). Therefore, 2-B4O may increase the level of CRF and corticotropin. The finding that stress-induced suppression of LH secretion was reversed by administration of CRF antagonist suggests the concept that activation of an endogenous CRF pathway may interfere with reproductive function (30). The 3V administration of 2-B4O suppressed LH secretion most strongly. We demonstrated that pretreatment with 10 or 50 μg of CRF antagonist did not block the suppressive effect of 2-B4O on the secretion of LH. Our preliminary experiment revealed that pretreatment with CRF antagonist 30 min before 2-B4O administration did not block the suppressive effect of 2-B4O either. This finding suggests that 2-B4O may decrease the LH pulse not through CRF but through other factors.

In conclusion, these results indicate that 2-B4O impairs pulsatile LH release and that CRF is not involved in this suppressive effect. The suppressive effects of 2-B4O on the pulsatile secretion of LH in OVX rats may be contributory to delay of estrus in fertile rats. Further studies on the serum levels of 2-B4O in patients with anorexia nervosa are necessary to clarify the clinical significance of 2-B4O.

Acknowledgments. We are indebted to the NIIDDK and the National Hormone and Pituitary Program (University of Maryland School of Medicine) for supplying a rat LH RIA kit. This work was partly supported by a grant from the Research Group on Anorexia Nervosa, sponsored by the Japanese Ministry of Health and Welfare.

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Received April 5th, 1993
Accepted July 30th, 1993