—Original Article—

Central Injection of Ketone Body Suppresses Luteinizing Hormone Release via the Catecholaminergic Pathway in Female Rats

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Abstract. Ketosis is found in various pathophysiological conditions, including diabetes and starvation, that are accompanied by suppression of gonadal activity. The aim of the present study was to determine the role of ketone body in the brain in regulating pulsatile luteinizing hormone (LH) secretion in female rats. Injection of 3-hydroxybutyrate (3HB), a ketone body, into the fourth cerebroventricle (4V) induced suppression of pulsatile LH secretion in a dose-dependent manner in ovariectomized (OVX) rats with an estradiol (E2) implant producing diestrus plasma E2 levels. Plasma glucose and corticosterone levels increased immediately after the 4V 3HB injection, suggesting that the treatment caused a hunger response. The 3HB-induced suppression of LH pulses might be mediated by noradrenergic inputs to the hypothalamic paraventricular nucleus (PVN) because a local injection of α -methyl-*p*-tyrosine, a catecholamine synthesis inhibitor, into the PVN blocked 3HB-induced suppression of LH pulses and PVN noradrenaline release was increased by 4V 3HB injection in E2-primed OVX rats. These results suggest that ketone body sensed by a central energy sensor in the hindbrain may suppress gonadotropin release via noradrenergic inputs to the PVN under ketosis.

Key words: Energy, 3-Hydroxybutyrate, Ketosis, Noradrenaline

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Reproductive functions are known to be impaired under malnutrition in both sexes in several mammalian species [1–5]. Fasting, glucoprivation or lipoprivation has been well established to inhibit gonadal functions through suppressing pulsatile luteinizing hormone (LH) secretion in rats [6–12]. Malnutrition is often accompanied by a high level of circulating ketone bodies, which are by-products of enhanced fatty acid mobilization. For instance, glucoprivation causes enhanced fatty acid oxidation and ketosis [13]. Diabetes mellitus is a ketotic condition, and women with type 1 diabetes often show various reproductive problems [14, 15]. Dairy cows during early postpartum periods show a low LH pulse frequency concomitantly with an increase in 3-hydroxybutyrate (3HB), a ketone body, concentrations [16]. Thus, the suppression of gonadal functions under ketosis suggests a possibility that ketone bodies may function as a negative energy signal to suppress reproductive functions.

Changes in energy level might be detected by the brain to control reproductive functions as well as food intake [17]. Numerous studies have indicated that the brain energy sensor resides within the hypothalamus and brainstem [18–23]. Previous studies have raised the possibility that brainstem energy sensors sense glucose and fatty acid availability to regulate gonadotropin secretion because administration of 2-deoxyglucose (2DG), a competitive inhibitor of glucose oxidation, or mercaptoacetate (MA), an inhibitor of fatty acid oxidation, into the fourth cerebroventricle (4V)

suppresses LH pulses in rats [12, 24, 25]. Likewise, blockade of hindbrain monocarboxylate transporter 1 (MCT1), a ketone body transporter located in ependymocytes around the 4V, results in normalization of diabetic hyperphagia in rats [26]. We therefore hypothesize that ketone bodies are sensed by a brainstem energy sensor to control gonadal functions through regulation of gonadotropin secretion.

The present study tested if ketone body suppresses LH secretion through a specific neural pathway in female rats. To this end, the effect of 3HB injection into the 4V on pulsatile LH secretion was examined in female rats to investigate whether ketone bodies sensed by the hindbrain energy sensor act as a negative signal. We also examined whether noradrenergic inputs to the hypothalamic paraventricular nucleus (PVN) are associated with 3HB-induced pulsatile LH suppression because our previous studies showed that glucoprivic suppression of pulsatile LH suppression was mediated by noradrenergic inputs to the PVN [11] and that lipoprivation also suppressed pulsatile LH secretion via the PVN noradrenergic pathway [12, 25].

Materials and Methods

Animals

Adult female Wistar-Imamichi strain rats (Institute for Animal Reproduction, Ibaraki, Japan) weighting 230–270 g were housed at an ambient temperature of 24 ± 2 °C under a light/dark cycle of 14-h light and 10-h darkness (lights on 0500 h). The animals had free access to food and water except that they were deprived of food at the start of blood sampling. All surgical procedures were performed under isoflurane anesthesia and aseptic conditions.

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Animals having shown at least 2 consecutive 4-day estrous cycles were bilaterally ovariectomized (OVX) a week before blood sampling. The OVX animals immediately received a subcutaneous Silastic implant (inner diameter, 1.5 mm; outer diameter, 3.0 mm; length 25.0 mm; Dow Corning, Midland, MI, USA) containing estradiol-17 β (E2; Sigma-Aldrich, St. Louis, MO, USA) dissolved in peanut oil at 20 μ g/ml for 1 week to serve as the OVX+E2 group. The E2 implant produces plasma E2 levels found at diestrus [8]. The E2 treatment was chosen because it causes significant suppression of LH pulses when animals were subjected to 48-h fasting [8] and enhances glucoprivic suppression of LH pulses [27].

The present experiments were approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University.

Effect of 4V 3HB administration on LH pulses and blood glucose levels

One week before blood sampling, a stainless-steel guide cannula (23 gauge, Plastics One, Roanoke, VA, USA) was stereotactically implanted into the 4V in OVX+E2 rats, as previously described [24]. The tip of the guide cannula was placed 12.5 mm posterior and 8.0 mm ventral to bregma at midline.

An internal cannula (26 gauge, Plastics One) was inserted into the guide cannula on the day of blood sampling to allow administration of 3HB (DL- β -hydroxybutyric acid sodium salt, Sigma-Aldrich) into the 4V. The 3HB was freshly prepared in artificial cerebrospinal fluid (aCSF) at a dose of 4, 8 or 16 μ mol/2 μ l and administered with a microinfusion pump at a flow rate of 1 μ l/min for 2 min into the 4V of the unstrained rats. Control animals received aCSF. Blood sampling started immediately after 4V 3HB.

Blood samples (100 μ l) were collected for 3 h from 1300 h at 6-min intervals through an indwelling atrial cannula (silicon tubing, inner diameter, 0.5 mm; outer diameter, 1.0 mm; Shin-Etsu Polymer, Tokyo, Japan) that had been inserted the day before blood sampling through the right jugular vein. Each blood sample was replaced with an equivalent volume of washed red blood cells obtained from other rats to keep the hematocrit constant. Plasma samples were obtained by immediate centrifugation and stored at -20 C until assayed for LH in all experiments. Plasma glucose level was measured in an additional volume (50 μ l) of blood samples obtained at 12- and 30-min intervals during the first hour and last 2 h of the sampling period, respectively, because the increase in plasma glucose levels is one of the hunger responses. Plasma glucose levels were assayed immediately after blood sampling.

At the end of the blood sampling, the rats were anesthetized and infused with 3% brilliant blue dye solution at the same volume as the drug infusions to ascertain whether 3HB was infused into the 4V. Immediately after dye infusion, the rats were euthanized, and the brain was removed. Cannula placement and the infusion site were verified by visual inspection. Only the data from animals with appropriate cannula placement in the 4V were used.

Determination of noradrenaline release in the PVN after 3HB injection into the 4V with microdialysis

Microdialysis of the PVN was conducted in OVX+E2 rats as described previously [28] to determine if 4V 3HB infusion

increases noradrenaline (NE) release in the PVN. Briefly, a guide cannula (0.5 mm outer diameter, AG-12, Eicom, Kyoto, Japan) was stereotactically implanted unilaterally into the PVN. After a 7-day recovery period, a microdialysis probe (50 kDa cutoff, 2 mm in length, 0.22 mm outer diameter, A-I-12-02, Eicom) was inserted into the PVN through the guide cannula 2 h before the onset of dialysis. The PVN was perfused continuously through the probe with Ringer's solution (147 mM NaCl, 4 mM KCl and 2.3 mM CaCl₂) using a microinfusion pump (ESP-64, Eicom) at 1 μ l/min. Dialysate was collected into tubes containing 5 μ l of 0.02 N HCl on ice every 20 min for 5 h starting at 1100 h, and 3HB (4 μ mol or 16 μ mol/2 μ l) was infused into the 4V at 1300 h as described above. Control animals received aCSF infusion into the 4V.

At the end of the experiment, animals were infused with 3% brilliant blue into the PVN and 4V. They were then perfused with saline, followed by 10% formalin under deep anesthesia. Coronal sections of the brain including PVN were made at 50 μ m and stained with thionin. The placement of the PVN cannula was verified with a microscope. The placement of the 4V cannula was verified by visual inspection. Only the data from animals with correct cannula placement in the PVN and 4V were used.

Effects of blockade of catecholamine synthesis in the PVN on 3HB-induced changes in plasma LH, glucose and corticosterone levels

Alpha-methyl-*p*-tyrosine (AMPT), a catecholamine synthesis inhibitor, was injected into the PVN before 4V 3HB injection to determine whether 3HB-induced pulsatile LH suppression is mediated by PVN noradrenergic inputs. One week before blood sampling, two guide cannulas for microinjection into the PVN and 4V were stereotactically implanted in each OVX+E2 animal. A guide cannula (26 gauge, Plastics One) was unilaterally implanted into the PVN with its tip placed 1.9 mm posterior and 7.6 mm ventral to bregma and 0.5 mm lateral to midline. A guide cannula was implanted into the 4V as described previously.

The animals received PVN infusion of 50 μ g AMPT (Sigma-Aldrich) in 0.5 μ l saline or an equal volume of saline for 2 min at a rate of 0.25 μ l/min through an inner cannula (31 gauge, Plastics One) 3 h before the onset of blood sampling and 4V drug infusion. The dose of AMPT was chosen based on our previous study, in which a single infusion of the same dose of AMPT reversed LH pulses suppressed by acute fasting [9] and 4V MA administration [12].

Blood sampling started immediately after 4V 3HB (16 μ mol/2 μ l) or aCSF injection. This treatment regimen resulted in the following four groups of rats: PVN saline plus 4V aCSF, PVN saline plus 4V 3HB, PVN AMPT plus 4V aCSF and PVN AMPT plus 4V 3HB. Blood sampling was performed as described previously. Plasma glucose and corticosterone levels were also measured to determine whether 3HB-induced hyperglycemia was partly mediated by activation of the hypothalamo-pituitary-adrenal axis.

At the end of the experiment, the placements of the PVN and 4V cannulas were determined as described above in detail. Only the data from animals with correct cannula placement in the PVN and 4V were used.

Assays

Plasma LH concentrations were determined by double-antibody radioimmunoassay (RIA) with a rat LH RIA kit provided by the National Hormone and Peptide Program (Baltimore, MD, USA) and were expressed in terms of the NIDDK rat LH-RP-3. The least detectable level in 50- μ l plasma samples was 0.156 ng/ml, and the intra- and interassay coefficients of variation were 4.4% and 6.9% at 1.2 ng/ml, respectively.

Plasma corticosterone concentrations were determined by RIA using synthetic corticosterone (Sigma-Aldrich) as a reference standard. Rabbit anti-corticosterone antiserum and tritiated corticosterone were obtained from Teikoku Hormone Medical, Tokyo, Japan, and Amersham Life Sciences, Buckinghamshire, UK, respectively. Plasma samples (1 μ l) were extracted twice with diethyl ether. The least detectable concentration was 12.5 pg/ml, and the intra- and interassay coefficients of variation were 12.2% at 0.188 ng/ml and 9.1% at 0.345 ng/ml, respectively.

Plasma glucose concentrations were determined by the glucose oxidase method using a commercial kit (Glucose C-Test, Wako, Osaka, Japan).

An HPLC-electrochemical detector system was used to quantify NE levels in PVN dialysate as described previously [11]. The system consisted of a reverse-phase column (Eicompack CA-5ODS, outer diameter 2.1 mm and 150 mm in length, Eicom) and an electrochemical detector (ECD300, Eicom) with an oxidation potential of +450 mV. The mobile phase consisted of 0.1 M phosphate buffer (pH 6.0) containing EDTA-2Na (50 mg/l), sodium 1-octanesulfonate (400 mg/l) and 5% methanol. The standard noradrenaline (L-Noradrenaline) was obtained from Nacalai Tesque, Kyoto, Japan. The least detectable NE level was 1.25 pg, and the intra- and interassay coefficients of variation were 5.6 and 7.4%, respectively.

Data analysis

All data are reported as means $\pm$ SEM. LH pulses were identified with the PULSAR computer program [29] as previously described [30]. Statistical differences in mean LH concentration and the frequency and amplitude of LH pulses (Fig. 1B) were determined by one-way ANOVA followed by Fischer's PLSD test. Statistical differences in plasma glucose and corticosterone levels (Figs. 1C, 3C and 3D) and in PVN NE release (Fig. 2) between groups were analyzed by two-way ANOVA (treatment and time as main effects) followed by the Bonferroni test. Statistical differences in mean LH concentration, frequency and amplitude of LH pulses (Fig. 3B) among groups were determined by two-way ANOVA (PVN treatment and 4V treatment as main effects) followed by the Bonferroni test.

Results

Effects of 4V 3HB administration on pulsatile LH secretion and blood glucose levels

The infusion of 3HB into the 4V suppressed pulsatile LH release in a dose-dependent manner in OVX+E2 rats (Fig. 1A), resulting in a significant ($P < 0.05$, Fischer's PLSD test, Fig. 1B) decrease in mean LH levels in animals with 8 or 16 μ mol 3HB infusion com-

Fig. 1. Effect of ketone body injection into the 4V on pulsatile LH secretion. **A:** Plasma LH profiles in representative OVX+E2 rats injected with aCSF or 3HB at a dose of 4, 8 or 16 μ mol into the 4V. Blood samples were collected immediately after 4V 3HB injection for 3 h at 6-min intervals. Arrowheads indicate the peaks of LH pulses identified by the PULSAR computer program. **B:** Mean plasma LH concentrations for 3 h and the frequency and amplitude of LH pulses in OVX+E2 animals treated with vehicle or 3HB into the 4V. Values are means $\pm$ SEM. The numbers in each column represent the numbers of animals used. Values with different letters are significantly different from each other ($P < 0.05$, one-way ANOVA followed by Fischer's PLSD test). **C:** Change in mean glucose levels after 4V 3HB injection in OVX+E2 rats. Statistical differences were determined by two-way ANOVA (treatment and time) followed by the Bonferroni test. *, ** and ***: $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. vehicle-treated controls, respectively. Values are means $\pm$ SEM.

pared with vehicle-treated controls. The frequency of LH pulses was significantly suppressed by 4V 3HB infusion at 16 μ mol. LH pulse amplitude was significantly suppressed by 4V 3HB infusion at any dose.

The plasma glucose level was increased by 4V 3HB administration in a dose-dependent manner (Fig. 1C). The highest dose of

Fig. 2. NE release in the PVN after ketone body injection into the 4V. Injection of 3HB into the 4V was performed at 1300 h immediately after sampling. The dialysate was collected every 20 min, and the NE content was expressed as a percent change from the preinjection value. Statistical differences were determined by two-way ANOVA (treatment and time) followed by the Bonferroni test. * and **: $P < 0.05$ and $P < 0.01$ vs. the preinjection value, respectively. Values are means $\pm$ SEM ($n = 4-6$).

3HB (16 μ mol) significantly increased plasma glucose levels throughout the 3-h sampling period except for at 48, 90 and 180 min after 3HB infusion compared with the vehicle-treated controls. The middle dose of 3HB (8 μ mol) caused a transient increase in plasma glucose concentrations, resulting in significant increases at 0 (immediately after infusion), 12 and 24 min after 4V infusion. The lowest dose of 3HB (4 μ mol) did not cause a significant increase in plasma glucose levels.

PVN NE release by 4V 3HB injection

Infusion of 4V 3HB caused an increase in NE release in the PVN in a dose-dependent manner (Fig. 2). NE release began to increase immediately after the higher dose of 3HB (16 μ mol) infusion and reached its peak 40 min after infusion (Fig. 2). The NE increase induced by 16 μ mol of 3HB remained throughout the 3-h sampling period, resulting significantly ($P < 0.05$) higher levels compared with the preinjection value except for at 20, 60 and 180 min after 3HB injection. The lower dose of 3HB (4 μ mol) tended to increase NE release in the PVN after the 3HB infusion but did not have a significant effect.

Effects of PVN catecholamine synthesis blockade on 3HB-induced change in LH pulses and plasma glucose and corticosterone levels

Pretreatment of animals with PVN AMPT injection blocked the suppressive effect of 4V 3HB injection on LH pulses (Fig. 3A). PVN AMPT injection alone did not affect pulsatile LH secretion in animals with 4V vehicle injection.

Mean LH levels and the frequency and amplitude of LH pulses were significantly suppressed by 4V 3HB injection ($P < 0.05$, Bonferroni test, Fig. 3B). Injection of 3HB did not suppress mean LH

levels and the frequency when the animals were pretreated with PVN AMPT ($P < 0.05$, Bonferroni test, Fig. 3B). The amplitude of LH pulses was significantly suppressed by 4V 3HB injection compared with the 4V vehicle-treated controls in the PVN AMPT-pretreated animals ($P < 0.05$, Bonferroni test, Fig. 3B).

Plasma glucose levels immediately increased after 4V 3HB injection in the PVN vehicle-pretreated animals. The increased glucose levels were significantly higher compared with the 4V vehicle-treated controls pretreated with AMPT or vehicle in the PVN (Fig. 3C). PVN AMPT pretreatment had no apparent effects on the 3HB-induced increase in the plasma glucose level, resulting in the glucose level being significantly higher than in the control animals without 4V 3HB treatment. Likewise, plasma corticosterone levels significantly increased after 4V 3HB injection in the PVN vehicle-pretreated animals compared with the 4V vehicle-treated controls (Fig. 3D). The 4V 3HB-induced corticosterone increase was not blocked by PVN AMPT preinjection. Control animals with 4V aCSF injection presented regular LH pulses and normal levels of plasma glucose or corticosterone regardless of PVN AMPT treatment.

Discussion

The present study demonstrated that 4V administration of 3HB, a ketone body, suppressed pulsatile LH release and induced an increase in plasma glucose and corticosterone levels in the rat. This result suggests that ketone bodies sensed in the hindbrain could function as a negative energy signal to inhibit gonadotropin secretion under ketotic conditions, such as diabetes or lactation. The present study also showed that ketone body-induced LH suppression is mediated by noradrenergic input to the PVN because 4V 3HB administration increased NE release in the PVN and inhibition of catecholamine synthesis in the PVN by local injection of AMPT overrode the 4V 3HB-induced suppression of LH pulses.

It has previously been proposed that hindbrain ependymocytes sense glucose levels to control feeding and reproduction. Administration of 2DG into the 4V increases food intake and suppresses LH secretion in rats [24]. Pancreatic glucokinase, an enzyme playing a key role in glucose sensing in pancreatic B cells, is found in 4V ependymocytes [31]. Intracellular calcium levels in the hindbrain ependymocytes respond to the change in extracellular glucose levels [23]. Hindbrain ependymocytes also might sense fatty acids because 4V MA administration inhibits pulsatile LH secretion [12]. Monocarboxylate transporter 1, a ketone body transporter, is expressed in ependymocytes around the 4V [26] as well as the lateral ventricle and third ventricle [32, 33], and blockade of hindbrain MCT1 reverses diabetic hyperphagia in rats [26]. Plasma 3HB levels positively correlate with CSF 3HB levels [26]. Hindbrain ependymocytes, therefore, may be equipped with an energy sensor monitoring levels of energy substrates, such as glucose, fatty acid and ketone body, and integrate the information on energy substrates to control feeding and gonadotropin secretion. The mechanism of energy sensing is largely unknown, but there might be some clues for the mechanism. For instance, 3HB can compete with pyruvate for oxidative metabolism in brain tissue [34]. Pyruvate oxidation is inhibited by 3HB in isolated cerebrocortical mitochondria mainly through phosphorylation of the

Fig. 3. Effect of inhibition of catecholamine synthesis in the PVN on ketone body-induced LH suppression. **A:** Representative plasma LH profiles from OVX+E2 rats injected with 3HB (16 μ mol) or vehicle into the 4V and receiving a preinjection of either AMPT or vehicle into the PVN. AMPT or vehicle was administered 3 h before the start of blood sampling. Injection of 3HB or vehicle was performed immediately before the start of blood sampling. Arrowheads indicate the peaks of LH pulses indicated by the PULSAR computer program. **B:** Mean plasma LH concentrations for 3 h and the frequency and amplitude of LH pulses in OVX+E2 rats treated with a 3HB or vehicle into the 4V and an AMPT or vehicle preinjection into the PVN. Values are means $\pm$ SEM. The numbers in each column represent the numbers of animals used. Statistical differences were determined by two-way ANOVA (PVN treatment and 4V treatment) followed by the Bonferroni test. * and **: $P < 0.05$ and $P < 0.01$ vs. 4V vehicle-treated controls with the same PVN pretreatment animals, respectively. # $P < 0.05$ vs. PVN vehicle-treated controls with the same 4V treatment animals. **C:** Changes in mean plasma glucose levels after 3HB or vehicle injection into the 4V in OVX+E2 rats receiving a preinjection with AMPT or vehicle into the PVN. Statistical differences were determined by two-way ANOVA (treatment and time) followed by the Bonferroni test. *, ** and ***: $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. 4V and PVN vehicle-treated animals, respectively. Values are means $\pm$ SEM. **D:** Changes in mean plasma corticosterone levels after 3HB or vehicle injection into the 4V in rats treated with a preinjection of AMPT or vehicle into the PVN. Statistical differences were determined by two-way ANOVA (treatment and time) followed by the Bonferroni test. *, ** and ***: $P < 0.05$, $P < 0.01$ and $P < 0.001$ vs. 4V and PVN vehicle-treated animals, respectively. Values are means $\pm$ SEM.

pyruvate dehydrogenase complex, which catalyzes the oxidative decarboxylation of pyruvate in mitochondria [35]. Thus, ketone bodies may be associated with the inhibition of glucose metabolism in ependymocytes to sense the level of energy substrates. Further studies are required to address these points.

Previous studies suggested that LH pulse suppression caused by malnutrition, such as fasting [9], glucoprivation [11] or lipoprivation [12, 25], is mediated by noradrenergic neurons projecting to the

PVN. Local PVN injection of $\alpha 1$ - or $\alpha 2$ -adrenergic receptor agonists suppressed pulsatile LH release in female rats [10]. Lipoprivic suppression of LH pulses is rescued by pretreatment with $\alpha 1$ - or $\alpha 2$ -adrenergic receptor antagonists in the PVN [25]. Thus, PVN $\alpha 1$ - or $\alpha 2$ -adrenergic receptor may be associated with the suppressed gonadotropin release under decreased energy availability. Likewise, 3HB-induced LH suppression was also rescued by blockade of catecholamine synthesis in the PVN in the present study. Taken together,

catecholaminergic inputs to the PVN might be a common pathway mediating the suppression of LH secretion under malnutrition.

The present study showed that 4V 3HB injection increased plasma glucose and corticosterone levels, while 3HB-induced glucose and corticosterone increases were not blocked by PVN AMPT preinjection. Therefore, the 3HB-induced increase in plasma glucose and corticosterone levels may be induced by other pathways, such as a sympathetic activity, not the PVN noradrenergic pathway. This is consistent with a previous report demonstrating that plasma noradrenaline levels are increased by ketone body administration [26].

In conclusion, the present study proposed that ketone bodies, which are overproduced under severe energy deficiency, might function as a negative energy signal to inhibit gonadal functions through suppression of gonadotropin secretion. Ketone bodies in the CSF, which reflect plasma levels, are likely to be sensed by a hindbrain energy sensor, most probably the ependymocytes, and the information is likely relayed by noradrenergic inputs to the PVN to regulate gonadotropin release.

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