Effect of Melatonin Injected into the Third Ventricle on Growth Hormone Secretion in Holstein Steers

Etsuko KASUYA¹⁾, Shiro KUSHIBIKI²⁾, Madoka SUTOH²⁾, Toshiyuki SAITO¹⁾, Shuichi ITO¹⁾, Ken-ichi YAYOU¹⁾, Ryosuke SAKUMOTO¹⁾ and Koichi HODATE³⁾

¹⁾Department of Physiology and Genetic Regulation, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305–8602,

²⁾Department of Animal Physiology and Nutrition, National Institute of Livestock and Grassland Science, Tsukuba, Ibaraki 305–0901 and

³⁾School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034–8628, Japan

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ABSTRACT. The effects of melatonin (MEL) injection into the third ventricle (3V) on growth hormone (GH) secretion were investigated in conscious Holstein steers. A stainless steel cannula was stereotactically implanted in the 3V based on the ventriculogram. In Exp. 1, three doses of MEL (100, 300 or 600 μ g) were injected into the 3V through the cannula and the GH concentration after the injection was determined. In Exp. 2, intracerebroventricular (icv) and intravenous (iv) injections of MEL (100 μ g) and GH-releasing hormone (GHRH; 0.25 μ g/kg body weight), respectively, were performed simultaneously to examine the effect of MEL on GHRH-induced GH release. The icv injection of MEL significantly stimulated GH release at 100 μ g. The increase in GH concentrations by 100 μ g of MEL was persistent. Intravenous injection of GHRH dramatically increased GH release. The injection of MEL did not alter GHRH-induced GH release. These results suggest that MEL stimulates GH secretion possibly through the hypothalamus in cattle.

KEY WORDS: cattle, melatonin, somatotrophic axis, stereotactic, third ventricle.

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Growth hormone (GH) is a very important factor for livestock production [6]. GH-releasing hormone (GHRH) and somatostatin (SS) from the hypothalamus are primary factors controlling GH secretion in mammals. In addition to GHRH and SS, many neuromodulators have been considered to be involved in GH release regulation in many species. Although the central regulatory mechanism of GH secretion has been studied in sheep using hypophyseal portal blood sampling technique [4], such information is not well obtained in cattle.

The evidences have shown the possibility that melatonin (MEL) from the pineal gland is one of those factors controlling GH secretion; however, the exact effect of MEL on GH secretion is controversial. For example, in humans it was reported that MEL induced an increase in GH levels [27, 29]; however, other groups obtained no effects of MEL on GH secretion [13, 31]. The intramuscular injection of MEL decreased GH secretion in prepubertal boys and the same dose of MEL increased GH level in some pubertal subjects [17]. *In vitro* experiments showed that MEL reduced GH secretion from rat pituitary cells [8]. These variations of the effects of MEL on GH secretion could be caused by the differences in the experimental and physiological conditions, and there might be inter-species differences regarding the role of MEL in the regulation of GH secretion.

The purpose of the present study was to determine the role of MEL in the regulatory system of GH secretion in cattle. Since MEL has been reported to stimulate GH release via the hypothalamus [23, 29], the technique for the direct injection of MEL into the third ventricle (3V) at three doses (100, 300 and 600 μ g) was used. We also assessed whether MEL affects GHRH-induced GH release to reveal the involvement of the hypothalamus in GH secretion modu-

lated by MEL.

MATERIALS AND METHODS

Animals: Eleven Holstein steers (7 to 8 mo old at the time of surgery) raised on the farm of the National Institute of Livestock and Grassland Science (Tsukuba, Japan) were used in the present study. The animals were fed hay and concentrate twice a day at 0830 and 1500 and allowed water *ad libitum*. Each animal was tied with a stanchion stall and its rectal temperature was monitored just before each feeding every day. The surgical and experimental procedures used on the animals were approved by the Institute Committee for Animal Use and Care at the National Institute of Agrobiological Sciences.

Stereotaxic surgery: Animals were deprived of food on the day before brain surgery. After the animal was sedated with an intramuscular injection of xylazine hydrochloride (Ceraclal, Bayer Japan, Tokyo Japan, 112 mg/animal at the maximal level and actual dose was depending on the animal's condition), tracheal intubation was performed. Then, inhalational general anesthesia was initiated with isoflurane (Isoflu, Abbott Laboratories, North Chicago, IL, U.S.A.) and nitrogen monoxide (0.5 l/min). The detail design of the stereotaxic apparatus for cattle [24] was already described. The cerebral ventriculography was performed based on the previous report [12] with small modification. The head of the animal was mounted on the apparatus properly, and the surface of the skull was exposed. Then a small hole was created on the skull to insert a spinal needle (18 gauge, Terumo, Tokyo, Japan) into the lateral ventricle of the brain. Radio-opaque dye (Iopamiron 300, Nihon-Schering, Osaka, Japan) was injected into the lateral ventricle through the needle and

Fig. 1. Representative lateral ventriculogram obtained from the stereotaxic surgery in Holstein steers. The letter "X" indicates the position of the tip of implanted cannula. Note that the cannula was exactly inserted into the third ventricle (3V).

X-ray pictures were taken to visualize the 3V. According to those images, the exact position of the third ventricle was expressed with 3-dimensional coordinates of the apparatus and a guide cannula (18 gauge, Eicom, Kyoto, Japan) was inserted until the tip of the cannula reached the 3V. The target point of the implantation is shown in Fig. 1. The cannula was fixed with dental acrylic resin (ADFA, Shofu Inc., Kyoto, Japan) and a stylet was inserted into the guide cannula until the day of the experiment. To recover the animal from anesthesia, an intramuscular injection of atipamezole hydrochloride (Antisedan, Meiji Seika Kaisha, Ltd., Tokyo, Japan) was performed. The steer was transferred from the surgery room into the experimental stall after the awakening and given approximately 10 days for its recovery.

Experiment 1: The effects of the intracerebroventricular injection of MEL on the secretion of GH in Holstein steers.

In Exp. 1, the effect of a single intracerebroventricular (icv) injection of MEL at three doses on the basal GH secretion was investigated. The animals were kept in individual indoor stalls under natural lighting conditions (10–13 hr of light) through the window. Mean room temperature was $20.9 \pm 0.6^\circ\text{C}$. The animals were well accustomed to the experimental conditions and researchers by the day of the experiment, such that the experimental procedures did not disturb the physiological conditions of the animal. An ind-

welling catheter was inserted into the external jugular vein at least 20 hr before the initiation of the experiment. At 1030, the animal was lightly restrained with a rope and the remaining feed was removed. Melatonin (Sigma, St. Louis, MO, U.S.A.; 100 μg or 300 μg in 200 μl 1% ethanol–0.9% NaCl) or vehicle (400 μl 1% ethanol–0.9% NaCl) was injected into the 3V via the implanted cannula at 1200 (0 min). For the dose of 600 μg , 400 μl of "300 μg solution" was injected. The icv injection was performed at the rate of 200 $\mu\text{l}/30$ sec. Blood samples were drawn at –60, –40, –20, 2, 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160 and 180 min after the icv injection. Each treatment (vehicle, 100, 300 or 600 μg of MEL) was randomized in each animal. Since the injection took 30 to 60 s, only the drawing immediately after the injection was performed with a 2-min delay. The blood (10 ml) was collected into the heparinized tubes and immediately put on ice. The plasma was separated by centrifugation ($1500 \times g$, 4°C , 30 min) after each experiment was finished and stored at -20°C until assayed.

Experiment 2: The effect of the intracerebroventricular injection of MEL on GHRH-induced GH secretion in Holstein steers.

In Exp. 2, the effects of icv injection of MEL on GHRH-induced GH secretion were investigated. This series of experiments was performed in the experimental room without windows under the artificial lighting (0600–1800; light), room temperature (20°C) and humidity (60%) conditions. The time course and procedure of blood drawing were the same as the ones of Exp. 1. Based on the result from Exp. 1, MEL (100 μg) was injected into the 3V through the implanted cannula at 0 min. Bovine GHRH (generously provided by Prof. Shin-ichi Ohashi, Kanazawa Institute of Technology, Japan; 0.25 $\mu\text{g}/\text{kg}$ body weight) diluted with sterilized 0.9% NaCl was injected intravenously, immediately before the blood sample (10 ml) was taken at 2 min after the icv injection. As a control for GHRH injection, 0.9% NaCl alone was administered intravenously. The data obtained from Exp. 1 was used for further comparison between MEL (100 μg) alone and GHRH with the MEL group.

Hormone assays and statistical analysis: Plasma GH concentrations were determined by double-antibody radioimmunoassay (RIA) based on the method of Johke [10]. The intra- and inter-assay CV was 6.3 and 9.4%, respectively. The assay sensitivity was 1.25 ng/ml. Plasma MEL concentrations were measured using a commercially available RIA kit (Bühlmann Laboratories, Schönenbuch, Switzerland) following the manufacturer's protocol. The intra- and inter-assay CV was 7.2 and 11.0%, respectively. The assay sensitivity was 0.5 pg/ml.

As an index of the amount of GH released in response to each agent, the area under the GH response curve (AUC) for 170 min after the injection was calculated. Data were represented as the mean $\pm$ standard error. The effects of MEL and GHRH on mean levels and AUCs of MEL and GH were determined using 1-way ANOVA, followed by *post hoc* analysis with the Tukey's test to compare the difference

between the values of control and treated groups. Statistical significance was attained at $P < 0.05$.

Macroscopic confirmation of the injection point: At the termination of experiments, the animal was euthanized by the injection of an overdose of sodium pentobarbital (Nembutal, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan). Approximately 100 μ l of brilliant blue solution was slowly injected through the implanted cannula just after the death of the animal to dye and visualize the injection point. When the cannula was properly implanted in the 3V, the inside wall of the 3V should have been stained blue. In the present study, the cannula was appropriately implanted in all cases, as confirmed macroscopically by this method.

RESULTS

Experiment 1: The effects of intracerebroventricular injection of MEL on the secretion of GH in Holstein steers.

The changes in plasma MEL concentrations after the icv injection of MEL are shown in Fig. 2. Plasma MEL levels were significantly increased immediately after the MEL injection at three doses applied (100, 300 or 600 μ g). The increase was observed in the plasma taken at 2 min after the injection and lasted until 120 or 180 min after the injection of 300 or 600 μ g of MEL, respectively, compared to the corresponding value of the control group. The some values of MEL between 2 and 50 min or 2 and 80 min after the injection of 300 or 600 μ g of MEL, respectively, were higher than those of the assay limitation, such that the actual MEL peak levels by 300 and 600 μ g injections were not obtained in the present study. The injection of MEL at 100 μ g induced smaller increase in MEL concentrations than that at 300 and 600 μ g. The peak level of plasma MEL was observed at 10 min after the icv injection and the significant increase in MEL level lasted until 80 min after the injection. On the other hand, the icv injection of vehicle (1% ethanol) did not cause any changes in the MEL concentration.

Figure 3A shows the changes in plasma GH concentrations after the icv injection of MEL at 3 doses. Although MEL injection tended to increase GH secretion at all doses examined, the significant increase was observed only in 100 μ g-treated group when the comparison was performed between control and MEL-treated group. The GH levels at 80 and 100 min after the icv injection of MEL (100 μ g) were higher than those of control group ($P < 0.05$). The tendency of the increase in GH concentrations was started just after the icv injection of MEL (100 μ g) and it lasted during the experimental period. The injection of the vehicle did not alter GH secretion. Figure 3B shows the AUC for 170 min after each treatment. The AUC for 100 μ g of MEL-treated group was significantly ($P < 0.01$) greater than that for controls.

Experiment 2: the effects of the intracerebroventricular injection of MEL on GHRH-induced GH secretion in Holstein steers

Figure 4A shows the changes in plasma GH concentrations after the iv injection of GHRH (0.25 μ g/kg body

Fig. 2. Changes in the plasma melatonin (MEL) concentration after intracerebroventricular (icv) injection of MEL (100; closed circles, 300; open squares or 600 μ g; closed squares) or 1% ethanol (EtOH, open circles with dotted line). An arrow shows the time of the injection. Each value is expressed as the mean + standard error. The asterisks show the statistical significance compared to the corresponding value of the control (1% ethanol) group (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$).

weight) alone or in combination with the icv injection of MEL (100 μ g). The injection of GHRH induced a significant increase in GH. The level of GH began to increase just after the injection of GHRH. The GH level reached the peak at 18 min after the iv injection, then it returned to its basal level (mean value of pre-injection period) 80 min after the injection. When GHRH was injected with icv MEL, it also stimulated GH secretion (Fig. 4). There was no significant difference between responses of GH to GHRH alone and in combination with MEL. Figure 4B shows the AUC for 170 min after each treatment. Both AUCs for GHRH alone and GHRH with MEL were significantly greater than that for control group ($P < 0.01$). There was no significant difference between the AUCs for GHRH alone and GHRH with MEL.

DISCUSSION

The present study demonstrated that a single injection of MEL into the 3V induced an increase in basal GH concentrations in Holstein steers (Fig. 3). The result suggests the possibility that MEL is involved in the regulation of GH secretion from the anterior pituitary in cattle. The effects of MEL on GH secretion are not consistent and are still controversial at present. For example, the stimulatory effect of MEL on GH secretion was observed when MEL was administered in the morning [27, 29, 30] when endogenous MEL levels are low [13]. The administration of MEL had no effect on GH secretion when it was given at 1700 [13]. The inconsistency observed in humans may have occurred due to the difference of the administration method or physiological condition. In the present study MEL was injected at noon, when the endogenous MEL levels were low in both the

Fig. 3. A: Plasma GH response to icv injection of melatonin (MEL, 100; closed circles, 300; open squares or 600 µg; closed squares) or 1% ethanol (EtOH, open circles with dotted line). An arrow shows the time of the injection. B: Area under GH response curve (AUC) after the icv injection. Each value is expressed as the mean $\pm$ standard errors. The asterisks show the statistical significance compared to the corresponding value of the control (1% ethanol) group (**, $P < 0.01$, *, $P < 0.05$).

plasma and the cerebrospinal fluid (CSF) in calves [9]. When MEL was injected at 100 µg into the 3V, plasma MEL concentrations reached approximately 130 pg/ml (Fig. 2). This MEL level is similar to that observed during night time in calves [9]. Therefore, the injection method and doses are considered to be appropriate to evaluate the role of MEL in the central regulatory mechanism of GH secretion.

In order to understand the mechanism underlying MEL-induced GH release, the effect of MEL on GHRH-induced GH release in steers was investigated (Exp. 2). Since physiological increase in MEL concentrations was obtained in 100 µg of MEL injected steers in Exp. 1, we chose 100 µg of MEL for the icv injection for Exp. 2. Although the present result showed that intravenous injection of GHRH dramatically increased GH secretion in steers, the peak level after GHRH injection in the present study was smaller than that of Holstein heifers at the corresponding age (6 mo old) shown in the report by Shingu *et al.* [26]. The difference is

Fig. 4. A: Plasma GH response to intravenous (iv) injection of GHRH alone (closed circles) or in combination with icv injection of melatonin (MEL, 100 µg, open squares). Closed and open arrows show the time of the icv and iv injections, respectively. B: AUC after icv and iv injections of MEL and GHRH. Each value is expressed as the mean $\pm$ standard errors. The asterisks show the statistical significance compared to the corresponding value of the control (SAL, iv) group (**, $P < 0.01$, *, $P < 0.05$).

appeared to be caused by the sexual dimorphism of GH response to GHRH in cattle [1].

The present result raises one possibility that the icv injection of MEL stimulated GH secretion through the hypothalamus. The endogenous MEL, secreted from the pineal gland, is detected not only in the blood but also in the CSF in several species, such as non-human primates [22], sheep [25], goats [11] and cattle [9]. The existence of MEL in the CSF is very important, because MEL from the pineal gland is supposed to enter the CSF through the pineal recess [28], and would reach the peri-3V area, especially the hypothalamus, the presumable target site of MEL controlling the reproductive axis in sheep [16, 18]. At this point of view, the injection of MEL into the 3V is an appropriate approach in order to evaluate the contribution of the hypothalamus to the effects of MEL on pituitary hormone secretion. Further-

more, the injection method into the 3V has an advantage over the lateral ventricular injection, because the substances injected into the 3V would reach more local area, which is the presumable target of substances injected than those into the lateral ventricle. Definitely, the medial basal hypothalamus has been shown as the binding site of MEL in the bovine brain [3]. In terms of the regulatory mechanism of GH secretion, most of GHRH cells are located in the arcuate nucleus of the bovine hypothalamus [15]. It is possible that the exogenous MEL injected into the 3V reached the arcuate nucleus and stimulated GHRH release followed by the increase in GH. However, the present results could not give a solid evidence for this hypothesis. Because the icv injection of MEL into the 3V did not alter the GH response to intravenous GHRH injection (Fig. 4). The involvement of the hypothalamus in MEL-mediated GH secretion has been suggested in several reports. Richardson *et al.* [23] suggested that MEL stimulated SS release from rat hypothalamic explants. As far as we know, this report is only evidence directly showing the involvement of the hypothalamus in MEL-mediated GH release. Another group reported that MEL administration increased not only basal GH but also GHRH-induced GH release in humans [29]. They interpreted the result to mean that MEL inhibited SS release at the hypothalamus level.

On the other hand, it has been suggested that MEL acts on the pituitary directly to affect GH secretion. MEL reduced GH release from GH4 cells [8]. Nonno *et al.* [20] suggested that MEL receptors were absent or the density of the receptors was extremely low in the bovine hypothalamus. Therefore, we could not exclude the possibility that MEL induces GH release at the pituitary level. Moreover, it has been reported that ghrelin, an endogenous ligand for the GH secretagogue that stimulates GH release, was decreased by MEL treatment [19]. The result suggests that factors other than hypothalamic hormones are involved in the regulation of GH secretion by MEL. Although the effects of MEL on GH secretion has been examined, a consistent understanding has not been obtained to date. Further investigation is required to solve remained questions.

The MEL secretion has a distinct nocturnal rise in cattle [5, 9] likewise in other species [11, 13, 22]. It is well known that GH secretion also has a nocturnal increment in humans [7]. Therefore, it was hypothesized that MEL could be involved in the increase in GH during the night. However, such nocturnal GH augmentation has not been clearly reported in cattle [2, 14, 21] so far. Further investigation for the relationship between spontaneous MEL secretion from the pineal gland and the somatotrophic axis in this species is necessary.

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