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Notes Pharmacology

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Plasma progesterone concentrations in female Wistar rats after exposure to urine preparations with and without protease-treatment were measured to explore the effects of protease-sensitive pheromones on the endocrine state. Exposure to crude urine excreted from male rats induced an increase in the plasma progesterone concentration in female rats. The progesterone concentration of oestrous females increased with an increase in the protein concentration in urine samples. Exposure of females in the oestrous state to urine preparations treated with protease did not induce increases in plasma progesterone. These results suggest that the presence of a protease-sensitive component in male urine exerts an influence on the endocrine state of oestrous females.
Key Words: progesterone, rat, urine, vomeronasal system, pheromone

Introduction

Various gonadal functions of females are controlled by pheromones in many vertebrates. Pheromones have been found in saliva, skin gland secretions, and urine. For example, pheromones in the urine excreted from male and female rats induce various changes in gonadal function and endocrine state, including reflex ovulation in the absence of coitus and mounting[1)], and a reduction in the oestrous cycle of female rats from 5 to 4 days[2)], and oestrous synchrony among female rats housed together[3)]. The vomeronasal organ exists in many vertebrates for the purpose of receiving pheromones, which in turn affect sexual and social behavior[4,5)].

In a previous study, we demonstrated that the sensory neurons in the vomeronasal organ (VNO) of female rats, which respond to male Wistar urine, are localized in the apical layer of the epithelium, where one type of $G_{i2\alpha}$ is selectively expressed[6)]. Exposure of the VNO of the female Wistar rat to male Wistar urine induces c-Fos expression, which is correlated with cellular activity, primarily in the rostral region of the accessory olfactory bulb (AOB)[7)], suggesting that pheromonal information received by the sensory neurons in the VNO is transmitted to neurons in the AOB. Exposure of the VNO of female rats to protease-sensitive urinary pheromones excreted from male rats induced the expression of c-Fos-immunoreactive cells in the AOB[8)]. In the present study, in order to characterize the effects of protease-sensitive pheromones present in male urine on the endocrine state of female rats, the plasma progesterone concentration in female rats was measured after the female rats had been exposed to male urinary pheromones with and without protease treatment.

Methods and Materials

Animals

All experiments were carried out in accordance with the Guidelines for the Use of Laboratory Animals of the Graduate School of Pharmaceutical Sciences, Hokkaido University. The Wistar rats were obtained from Sankyo Laboratory Co., Sapporo, Japan. The animals were housed in same-sex groups of four in a room that was maintained at a temperature of 22 ± 0.5°C, with a relative humidity of 58%, and on a 14-h light/10-h dark cycle (lights off at 21:00). The males and females were housed in the same room. All rats had free access to food and water. Three or four rats were subjected to each condition. The oestrous cycle was determined based on the observation of fresh vaginal smears of female Wistar rats (less than 6 months old) at 15:00. The following criteria were used to define the oestrous and dioestrous stages on a 4-day cycle: oestrous was identified by a smear of cornified cells, and dioesterous was identified by leucocytes in the presence of nucleated epithelial cells.

Stimulation with urine preparations

The noses of adult female rats from the Wistar strain were subjected to a spray of urine preparations from male Wistar rats or a control salt solution. The unanesthetized rats were gently held by hand for 50 min during the spraying of urinary samples (30 ml). Urine was collected from four or five one-year-old Wister rats using a metabolic cage. The protein concentration in the crude urine was 12.1 ± 3.8 mg/ml. The protein concentration in the urine preparation was adjusted to 5.0 mg/ml by dilution with the control salt solution, which has a similar ion composition to that of rat urine. The control salt solution consisted of (mM): 150 NaCl, 300 KCl, 1 $CaCl_2$, 3 $MgCl_2$, and 10 HEPES-NaOH[(pH 7.6). To explore the effects of protease-sensitive pheromones on

plasma progesterone, the urine containing 60 mM Tris (Tris (hydroxymethyl) aminomethane)-HCl (pH 7.0) was treated with 10 mg/ml pronase (type XIV, Sigma, St. Louis, MO for 60 min at 37°C.

Measurement of plasma progesterone

One hour after the exposure of the noses of the female rats to the urinary samples, 1 ml of blood was collected from a caudal blood vessel after the rats had been anesthetized with ether**.** The supernatant was used for the measurement of the concentration of progesterone in the plasma after the centrifugation (3000 x g, 15 min) of blood samples incubated for 4 h at 4°C. The plasma progesterone concentrations were measured by RIA using a commercial kit (ZB134, Diagnostic Products Corporation, San Diego, CA) according to the assay protocol supplied by the manufacturer. The plasma (100 μl) was incubated with ^{125}I-progesterone standard solution (1 ml) in a tube, and was used for binding to anti-progesterone antibody for 2 h at room temperature. The radioactivity of the tube after the solution had been discarded was measured in a scintillation analyzer γ-counter (WALLAC 1460SLR, Pharmacia, North Peapack, NJ).

Statistical analyses

A two-way ANOVA was used to test differences between the two groups after exposure to control salt solution and urine, and to test differences between cycles. One-way ANOVA was used to test differences between groups exposed to urine with different protein concentrations. One-way ANOVA was also used to test differences between groups with exposure to urine samples with and without the protease digestion.

Post-hoc analyses (Bonferroni/Dunn) were used to evaluate differences between values for each factor. Analyses were carried out using StatiView (SAS Institute Inc., Cary, NC).

Results

The effects of exposure of the vomeronasal organ to male urinary pheromones on the concentration of progesterone in the plasma were examined in female rats in both the dioestrous and the oestrous state (Fig. 1). Exposure to urinary pheromones excreted from male rats was associated with an increase in the plasma progesterone concentration of female rats in the dioesterous, as well as those in the oestrous state ($F(1, 12) = 18.5$, $P = 0.001$). However, exposure to urinary pheromones had no significant effect on the plasma concentration of esterone in either oestrous or dioesterous females (data not shown). The magnitude of increase in the plasma progesterone concentration after exposure of oestrous females to urine was more remarkable than that observed in females in the dioestrous state ($F(1, 12) = 4.7$, $P = 0.05$). Therefore, we analyzed the effects of urinary pheromone on the plasma progesterone concentration of oestrous female rats in subsequent experiments.

One-way ANOVA was used to compare the concentration of progesterone in the plasma after the female rats had been exposed to urine samples with different protein concentrations ($F(3, 9) = 17.3$, $P = 0.004$). The concentration of progesterone in the plasma increased with increases in the protein concentration in the urine samples used for exposure (Fig. 2). The concentration of progesterone in the plasma after the rats were exposed to diluted urine with 0.1 mg/ml protein was about twice the basal level, but this difference was not significant. Further increases in the protein concentrations

used in the urine dilution induced increases in the plasma concentration of progesterone ($P < 0.001$).

Figure 3 shows the plasma concentration of progesterone after exposure to urine preparations with and without pronase digestion. One-way ANOVA was used to compare the plasma concentration of progesterone after the rats were exposed to either protease-treated or protease-untreated urine samples ($F(2, 9) = 32.2$, $P < 0.001$). The plasma concentration of progesterone in oestrous females after they had been exposed to the urine preparation subjected to protease digestion was lower than that of oestrous females after exposure to the urine preparation not subjected to protease digestion ($P = 0.023$). The concentration of progesterone in the plasma after exposure to the protease-treated urine sample was higher than that after exposure to the control salt solution but this difference was not significant.

Discussion

Pheromones have been identified as proteins and as molecules of low molecular weight[9-12]. It has been demonstrated that the vomeronasal pump takes pheromones dissolved in fluid into the VNO[5]. For example, compounds of low volatility such as the major urinary protein complex and aphrodisin, which belongs to a family of extracellular proteins (lipocalin), induce the acceleration of the onset of puberty in mice and copulatory behavior in male hamsters via the VNO, respectively[13-15]. It is therefore possible that female rats are able to detect peptides in male rat urine as pheromones via the vomeronasal system

In the AOB, the mitral/tufted cells receive direct input from the vomeronasal sensory neurons at the glomeruli. The cell bodies of vomeronasal sensory neurons are

located at various depths in the cellular layer of the sensory epithelium. The sensory neurons at the apical and basal layers of the sensory epithelium of marsupials and rodents are immunoreactive to anti-$G_{i2\alpha}$ and anti-$G_{o\alpha}$ proteins, respectively[16,17]. The responses of the sensory neurons in the apical portion of the female rat vomeronasal epithelium to male Wistar urine were mediated via G_i, whereas the responses of the neurons in the basal portion of the female rat vomeronasal epithelium to male Donryu urine were mediated via G_o [6]. Two populations of sensory neurons in the vomeronasal organ project information to different regions of the glomerular layer of the AOB[17]. The rostral region of the AOB is innervated by a population of $G_{i2\alpha}$-expressing vomeronasal sensory neurons, the cell bodies of which are located in the apical layer of the vomeronasal sensory epithelium. The caudal region of the AOB glomerular layer is innervated by $G_{o\alpha}$-expressing vomeronasal sensory neurons[, the cell bodies of which] are located in the basal layer of the vomeronasal sensory epithelium[7].

No pheromones that induce behavioral and endocrinological changes in rats have been identified to date. The activity of a component in male urine that induced the expression of c-Fos-immunoreactivity in the caudal region of the AOB was abolished by papain treatment, whereas the corresponding activity in the rostral region was not abolished by treatment with papain[8]. Pronase treatment abolished the induction of immunoreactivity in the rostral region, as well as in the caudal region. These results suggest that at least two urinary peptides (i.e., papain-sensitive and papain-insensitive peptides) responsible for the stimulation of the vomeronasal organ of female rats are contained in male Wistar rat urine.

Exposure of the female rat vomeronasal organ to either the dialyzed urine preparation (< 500 Da) or the remaining substances (> 500 Da) in male rat urine did not

induce the expression of c-Fos-immunoreactive cells in the AOB, whereas exposure to a mixture of these preparations did induce such expression[18]. This result suggests that a combination of low molecular weight substances and high molecular weight substances, which are also likely to be protease-sensitive, is necessary for increases in c-Fos-immunoreactivity in the AOB.

Pheromones in urine have been indicated to induce changes in the endocrine system in rodents. In mice, pregnancy failed in a high population of mice when the mated female was exposed to odors of strange males of a different strain[19]. Plasma progesterone concentrations in copulated female mice increased from day 0 to day 4 after exposure to stud males[20]. However, the plasma progesterone concentrations in copulated females after exposure to strange males were lower that those after exposure to stud males. It is likely that changes in hormone concentrations induced by pheromones exert an effect on the persistence of pregnancy in mice. The pregnancy block has not been observed in rats. In rats, pheromones in the urine of males reduced the oestrous cycle of females from 5 to 4 days[2] and induced the dioestrous stage and restored oestrous cycles in irregularly cycling and anovulatory persistent-oestrous aging females[21]. Baseline plasma progesterone concentrations in irregularly cycling and anovulatory-oestrous females were similar to those in normally cycling 7-8-month-old females; male urinary pheromones increased the levels of plasma progesterone in these females[22]. In the present study, we demonstrated that urinary pheromones, which are sensitive to protease, increased plasma progesterone in females at the oestrous stage. It is likely that peptide pheromones affect gonadal function via the endocrine system in normally cycling females.

Acknowledgements

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Figure legends

Figure 1 Plasma progesterone concentrations in dioestrous and oestrous female rats after exposure to control salt solution open column) and urine (filled column). Values are the mean ± SEM (n = 4).

Figure 2 Plasma progesterone concentrations in oestrous female rats after exposure to urine with various protein concentrations. Values are the mean ± SEM. The number of animals is given in parentheses. *: $p < 0.001$.

Figure 3 Plasma progesterone concentrations in oestrous female rats after exposure to pronase-treated and pronase-untreated urine. Values are the mean ± SEM (n = 4).

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Figure 1

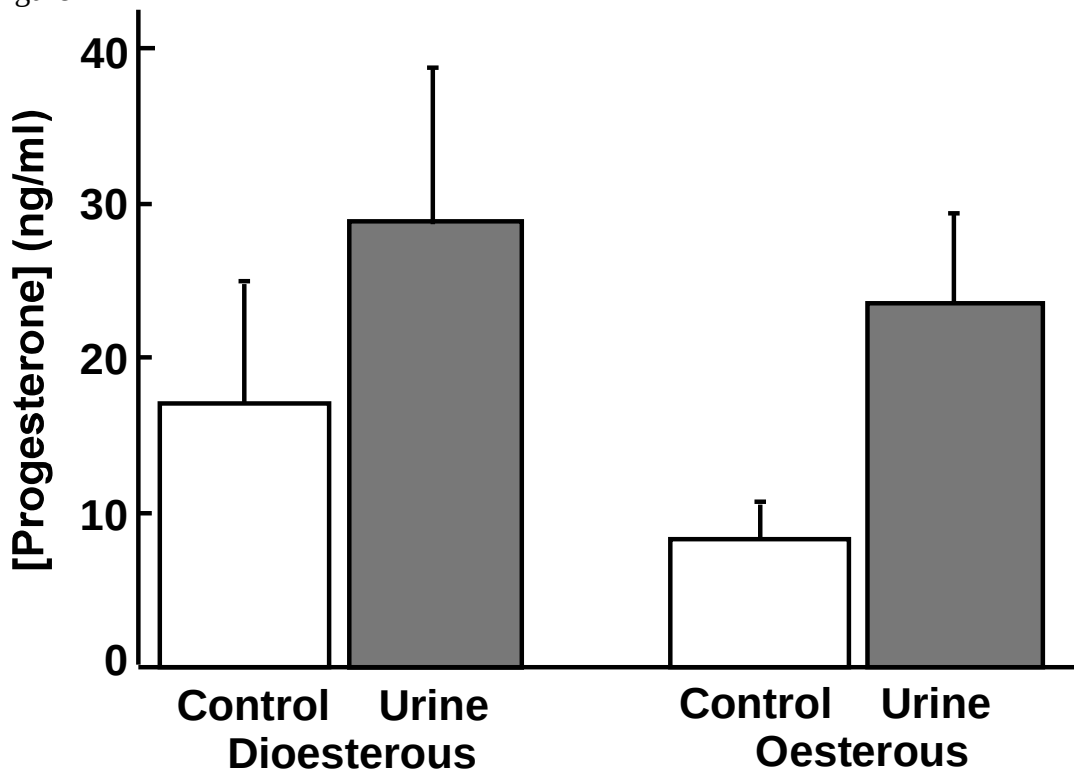

[Progesterone] (ng/ml)
40
30
20
10
0
Control
Urine
Dioesterous
Control
Urine
Oesterous

Figure 2

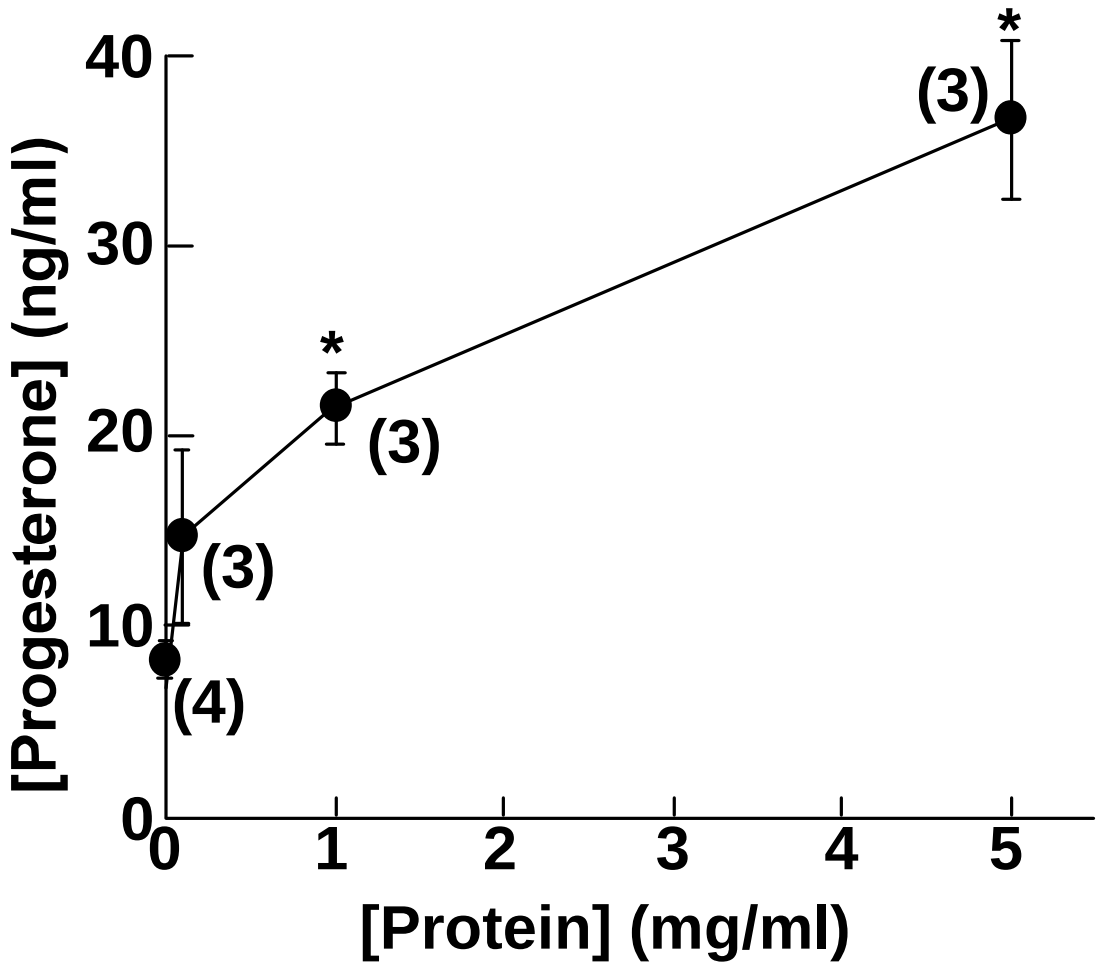
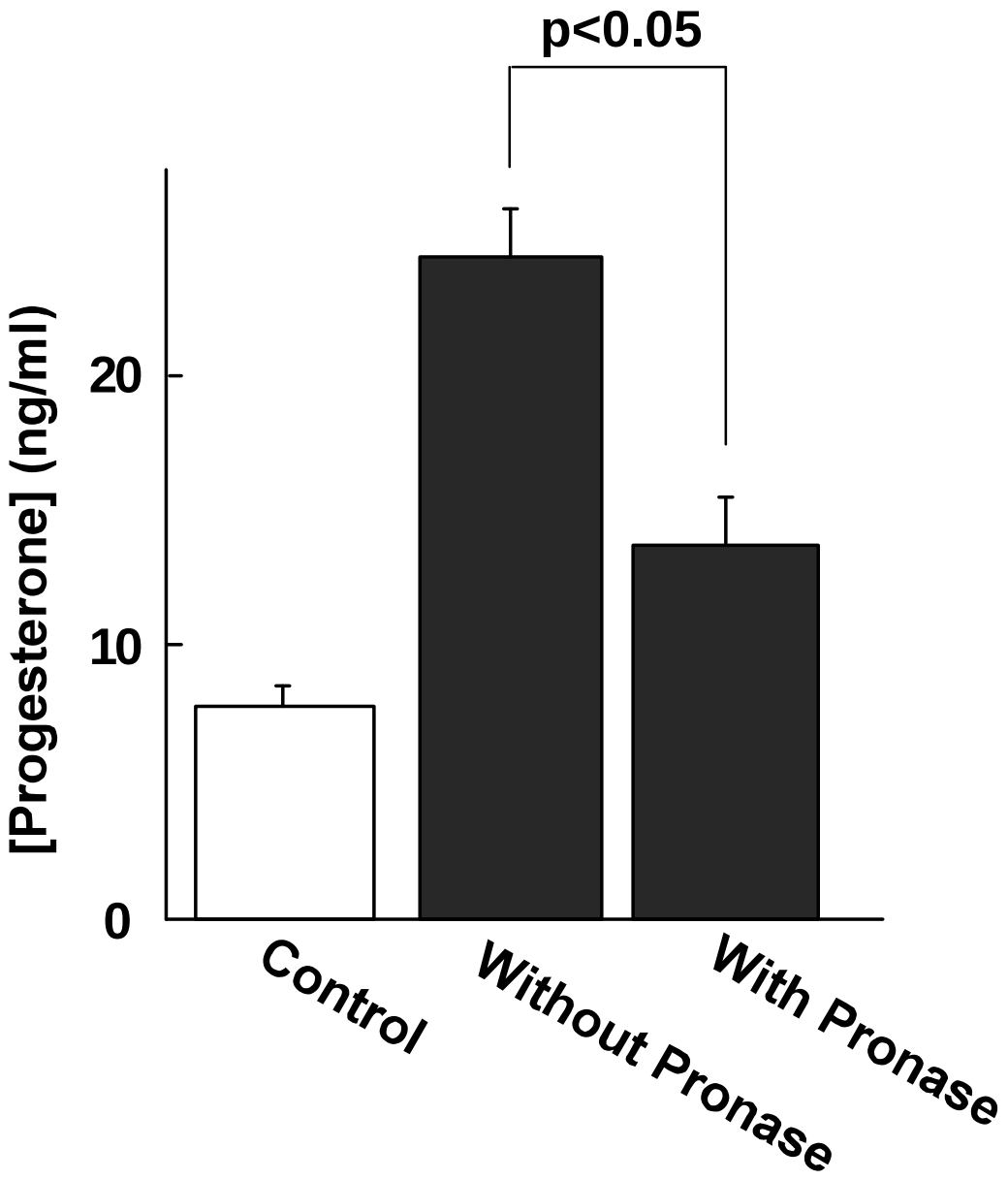


Figure 3


p<0.05
[Progesterone] (ng/ml)
20
10
0
Control
Without Pronase
With Pronase