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Ovariectomy-Induced Hyperalgesia and Antinociceptive Effect of Elcatonin, a Synthetic Eel Calcitonin

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SHIBATA, K., M. TAKEDA, A. ITO, M. TAKEDA, AND H. SAGAI. *Ovariectomy-induced hyperalgesia and antinociceptive effect of elcatonin, a synthetic eel calcitonin.* PHARMACOL BIOCHEM BEHAV **60**(2) 371–376, 1998.—Using ovariectomized (OVX) rats in the tail-withdrawal nociceptive test, we examined OVX-induced hyperalgesia and the antinociceptive effect of subcutaneously administered elcatonin, a synthetic derivative of eel calcitonin ([Asu^{1,7}] eel calcitonin). Because tail-withdrawal latency was significantly and continuously reduced and bone mineral density decreased in OVX rats compared with those of sham-operated rats, it was demonstrated that ovariectomy induced prolonged hyperalgesia and osteoporosis. After repeated administrations for 3 or 4 weeks, subcutaneously injected elcatonin increased the latency of the OVX rats in a dose-dependent manner, compared to the vehicle-treated OVX rats. At a dose of 20 U/kg/day, there were significant differences ($p < 0.01$) in the latency between the elcatonin- and vehicle-treated OVX rats. This effect of elcatonin was completely inhibited by *p*-chlorophenylalanine treatment, suggesting that the central serotonergic system may be involved in the elcatonin antinociception of OVX-induced hyperalgesia. © 1998 Elsevier Science Inc.

Ovariectomy-induced hyperalgesia Antinociception Calcitonin Elcatonin PCPA Serotonergic neuron Rat

CALCITONIN is a polypeptide hormone that is secreted from the parafollicular cells of the mammalian thyroid gland into general circulation (34,41). It regulates blood calcium concentration and bone metabolism by acting on osteoclasts (29,34,37, 47,53). Clinically, calcitonin is employed as a treatment to reduce blood calcium concentration in hypercalcemia (31,44) and as a treatment to improve bone mass in osteoporosis (6,16,38) and to relieve its accompanying pain (13,14,40).

It has been reported that calcitonin binding sites are densely distributed into the areas of the central nervous system (10,11,17,19,21,35,42,48), hypothalamus, brain stem, and spinal cord, which are intimately involved in the modulation of nociceptive transmission. In experimental studies using animals, it has been demonstrated that calcitonin has acute antinociceptive effects when administered intracerebroventricularly (2,7,8, 18,20,39,54) or intrathecally (18,45) or into periaqueductal gray matter (10), probably by acting on these binding sites directly. In some of these studies, the influence of opioidergic (10,45, 54), serotonergic (7,8,18), and catecholaminergic (8,18,20) antagonists on the antinociception of centrally injected calcitonin has been described, and as a matter of course, possible antinociceptive mechanisms have been proposed and discussed, but not yet standardized [see review, (4)]. Involvement of cholinergic (5) and NMDA (26) neurons in this effect has also been examined.

Intracardially injected calcitonin exclusively binds to the rat's circumventricular organs, which are blood–brain barrier free regions in the central nervous system, but not to the areas noted above, such as the hypothalamus [(50); our unpublished data]. This evidence suggests that calcitonin may not be able to pass through the blood–brain barrier, and that the antinociceptive effects examined by centrally injected calcitonin may not be produced by peripheral administration (2). Moreover, the acute antinociceptive effects of centrally administered cal-

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citonin do not seem to reflect the clinical features and effects of calcitonin because under clinical conditions calcitonin is administered to patients systemically, mainly intramuscularly, and a period of about a month is required for efficacy to appear in relieving the pain accompanying osteoporosis (13,14).

The antinociceptive effects of peripherally injected calcitonin using animals have been studied by two groups. Martin et al. (9,27,28) examined the acute antinociceptive effect of intraperitoneally injected calcitonin with the acetic acid writhing method in mice and with the tail-flick test in rats. The contributions of the opioidergic system in this antinociception were suggested by the interaction of calcitonin with morphine (27) and other opioidergic agonists (28). The participation of the serotonergic system was also suggested with the disappearance of the antinociceptive effect after administration of serotonin depletors, *p*-chlorophenylalanine (PCPA) and *p*-chloroamphetamine (9).

Umeno et al., the other group, were the first to report the antinociception of repeated SC injections of calcitonin on formalin-induced hyperalgesia in rats (49). Sequentially, using centrally administered depletors of monoamines (24) and antagonists of the serotonergic, catecholaminergic, and opioidergic systems (55), Kuraishi et al. investigated the involvement of these neuronal systems in the antinociception. The results obtained by Kuraishi et al. suggest that the ascending serotonergic system, rather than the noradrenergic, opioidergic, or descending serotonergic system, mediated the antinociception of formalin-induced hyperalgesia. Although Martin et al. and Kuraishi et al. have examined the antinociceptive effect of calcitonin by peripheral administration, their experiments seem to be insufficient because their nociceptive models may not correspond to the type of pain that accompanies osteoporosis.

In humans, it is well known that menopause is one of the essential causes of osteoporosis (1). The most important change following menopause is the depletion of estrous hormones such as estrogen, which regulates various gene expressions by forming a complex with the estrogen receptor followed by binding to a particular sequence in the promoter region of genes [see review, (3)]. Accordingly, it is easily anticipated that the depletion of this hormone influences the amount of gene products, including receptors and peptides, required for the modulation of nociceptive transmission. Such an alteration at the genetic expression level seems to be one of triggers for the pain accompanying postmenopausal osteoporosis.

These same hormonal changes that produce osteoporosis in humans also are observed in the ovariectomized (OVX) rat, making it the animal model routinely used for the evaluation of therapeutic agents for postmenopausal osteoporosis (52). Further, it has separately been reported that OVX in rats produces a significant reduction in the latencies for tail withdrawal from hot water, i.e., hyperalgesia (12). If osteoporosis and this type of hyperalgesia are simultaneously induced by OVX in rat, then the OVX rat becomes an ideal model for the investigation of the clinical analgesic effect of calcitonin.

In this first of a two-part series, we confirmed the manifestation of OVX-induced hyperalgesia and examined the antinociceptive effect of subcutaneously and repeatedly administered elcatonin (33) ([Asu^{1,7}] eel calcitonin], a synthetic derivative of eel calcitonin, using OVX rats with the tail-withdrawal nociceptive test.

It is well known that the central monoaminergic systems, such as the serotonergic and the noradrenergic, contribute to the modulation of nociceptive transmission (23,30,43,51,56). Moreover, as described above, the contribution of the serotonergic system in the antinociceptive effect of peripherally injected calcitonin has been suggested by two independent groups (9,24,55). Thus, in the second experiment described here, we also examined the involvement of the serotonergic system in the antinociceptive effect of elcatonin using PCPA (22), an inhibitor of serotonin biosynthesis.

METHOD

Animals

Female Sprague–Dawley rats obtained from Charles River Laboratory (Atsugi, Japan) weighing 180–200 g were used. Animals were individually housed in a temperature (22 ± 1°C) and humidity (55 ± 10%)-controlled room and in automatic breeding cages with a light–dark cycle (lights on at 0700 h, off at 1900 h), and with free access to food and water. Nociceptive tests were conducted during the light phase. To reduce stress, the animals were handled every day and adapted to a settling box about 24 h before each test.

Drugs

Elcatonin (33, Asahi Chemical Industry, Tokyo, Japan), was dissolved in vehicle (0.1 mM sodium acetate buffer (pH 5.5), 0.9% sodium chloride, 0.02% bovine serum albumin) and administered subcutaneously 5 times per week at a dose of 5 or 20 U/kg/day in a volume of 100 μl PCPA (22, Sigma) was dissolved in saline and injected intraperitoneally at a dose of 100 mg/kg/day, 72 and 24 h before the last tail-withdrawal test.

Surgery and Tail Withdrawal Test

In each experiment, 36 rats were surgically and bilaterally OVX under ether anesthesia and separated into three groups, and 12 rats were sham operated, in which the ovaries were exteriorized but not removed. All the rats were subcutaneously administered 100 μl of vehicle five times a week from 7 days before to 3 weeks after surgery because in a preliminary experiment it was observed that tail-withdrawal responses were influenced by injections when they were started midway through the experiment.

In Experiment 1, the first group of the 3 OVX groups was injected with 5 U/kg/day of elcatonin starting 3 weeks after surgery (at this point OVX had induced hyperalgesia). The second group was administered 20 U/kg/day of elcatonin, and the third OVX group and the sham group were continuously given vehicle.

In Experiment 2, starting 3 weeks after surgery, the first and the second OVX group were administered 20 U/kg/day of elcatonin, and the third OVX group and the sham group were injected with vehicle. The second group was intraperitoneally injected with PCPA at 8 weeks after surgery, and the other three groups were injected with saline instead of PCPA.

Each animal was mildly restrained in a settling box and its tail was immersed in a water bath maintained at a temperature of 50 ± 0.5°C. Measurements were made before surgery and once a week after surgery, and the latency required for the animal to withdraw its tail from the water bath was measured. The cutoff time was 7 s, but all rats tested responded within this period. Every tail withdrawal test was held over 4 h after the administration of elcatonin or vehicle.

Measurement of Bone Mineral Density

The right femur was ectomized from each rat in Experiment 1, and the muscles were removed. The bone mineral density of one-third of the distal portion was determined by dual-energy x-ray absorptiometry using an XR-26 instrument (Norland, USA).

Detection of Monoamine Content

All the rats in Experiment 2 were sacrificed by decapitation, and the left half of the brain and the lumbar spinal cord were rapidly removed. The tissues were weighed, the respective tissue from two rats were pooled into one sample, and the sample pools were homogenized in a cold solution containing 0.2 M perchloric acid, 0.05% EDTA, and 0.15% sodium metabisulfite. After centrifugation, the supernatant was adjusted to pH 3 by sodium acetate and analyzed using HPLC (Eicom ECD-100, column; Eicompak MA50SD, Japan) for serotonin, 5-hydroxyindoleacetic acid (5-HIAA), norepinephrine, and dopamine content.

Statistical Analysis

All results are presented as mean ± SEM. The significance of differences between groups was determined by the *t*-test or Dunnett's test. The probability level of $p < 0.05$ was considered to be statistically significant.

RESULTS

OVX Inducement of Hyperalgesia and Osteoporosis, and Dose-Dependent Antinociceptive Effect of Elcatonin

In Experiment 1, the tail withdrawal latencies of the sham group, but not of the OVX group, increased gradually after surgery compared with the presurgery latencies (Fig. 1. The latency of the vehicle-treated OVX group was significantly lower compared with that of the sham group 1 week after surgery, and significant differences in latency between the two groups were continuously observed during the experiment (Fig. 1), indicating that OVX induced prolonged hyperalgesia.

Figure 1 also shows the time course curves of the tail-withdrawal responses in the OVX rats following the SC injections of elcatonin. Comparing the elcatonin (20 U/kg/day)-injected OVX group with the vehicle-treated OVX group, the latencies of the elcatonin-injected group increased gradually. At 3 and 4 weeks of administration, significant differences in latency were observed between the elcatonin-treated (20 U/kg/day) and the vehicle-treated OVX groups ($p < 0.01$, Dunnett's test). At a dose of 5 U/kg/day, the latencies also increased, but the differences were not significant when compared to those of the vehicle-injected OVX group even at the end of the experiment (Fig. 1).

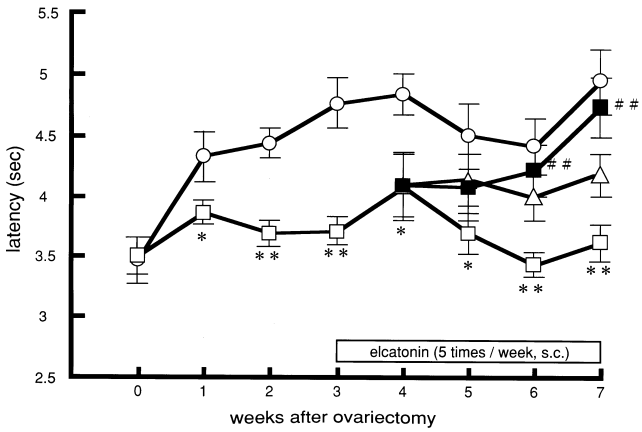

FIG. 1. Antinociceptive effects of elcatonin in the tail-withdrawal test of OVX rats. Thirty-six rats were OVX and 12 were sham operated. Tail-withdrawal latencies were measured once a week. OVX rats were divided into three groups, and vehicle (□), 5 U/kg/day of elcatonin (△) or 20 U/kg/day (■) was injected subcutaneously five times per week. Vehicle was injected to the sham (○). Each value is the mean of 12 data ± SEM. Significant difference; *$p < 0.05$, **$p < 0.01$ vs. sham, ##$p < 0.01$ vs. vehicle-injected OVX group.

As shown in Fig. 2, at the end of Experiment 1, the bone mineral density of the vehicle-treated OVX group decreased significantly compared with that of the sham ($p < 0.01$). This and the above results indicate that OVX induces both hyperalgesia and osteoporosis.

The decrease of bone mineral densities in OVX rats was slightly inhibited by the administration of elcatonin for 4 weeks (Fig. 2), although there is no significance among the 5 U, 20 U elcatonin-injected group and the OVX-vehicle group (Dunnett's test).

Serotonergic System Involvement in the Elcatonin Antinociception

In Experiment 2, the significant differences in the tail-withdrawal latencies were also observed between the vehicle-treated OVX group and the sham 1 week after OVX, and between the vehicle-treated and the elcatonin (20 U/kg/day)-treated OVX group on and after 4 weeks of administration of the drug (Fig. 3).

PCPA (100 mg/kg), an inhibitor of serotonin biosynthesis, was given twice, together with elcatonin (20 U/kg/day), to the elcatonin-treated OVX rats between 8 and 9 weeks after surgery. Those rats were exhibiting lasting, elcatonin-induced antinociception for the OVX-induced hyperalgesia. After the PCPA treatment, the latency decreased significantly ($p < 0.01$ vs. elcatonin/saline-injected OVX group, Fig. 3), similar to that of the vehicle/saline-injected OVX group, indicating complete abolishment of the antinociceptive effect of elcatonin by PCPA. In a preliminary experiment where PCPA alone was administered twice, the nociceptive response of the OVX rats was not influenced (data not shown).

Serotonin and Other Monoamine Levels in Spinal Cord and Cerebrum

We examined whether the monoamine levels in the spinal cord and in the cerebrum changed due to the OVX or the administration of elcatonin, using the rats in Experiment 2. As

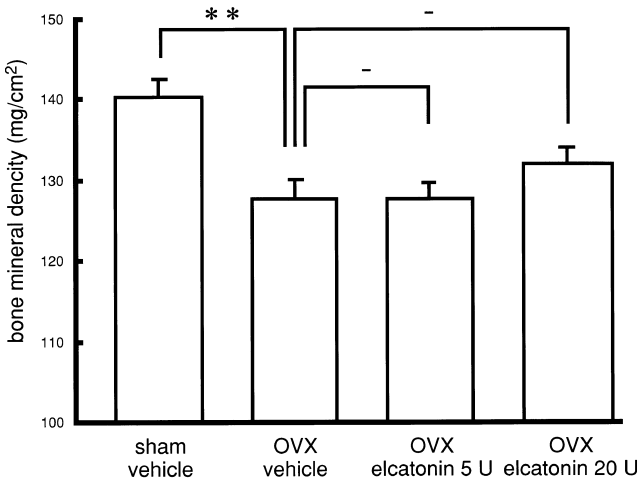

FIG. 2. Changes in bone mineral densities. Bone mineral densities of femur were measured by the dual-energy x-ray absorptiometry. Each bar represents the mean of 12 data ± SEM. Significant difference; **$p < 0.01$, – = not significant.

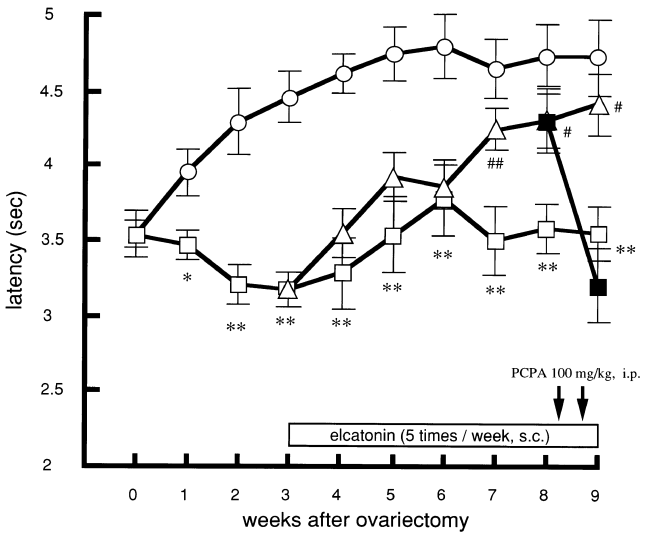

FIG. 3. Inhibitory effect of PCPA on elcatonin-induced antinociception. Rats were treated as described in Fig. 1. Each of the three OVX groups was injected with either vehicle (□), elcatonin (20 U/kg/day) and saline (△), or elcatonin (20 U/kg/day) and PCPA (100 mg/kg) (■). Vehicle was injected to the sham group (○). Each value is the mean of 12 data ± SEM. Significant difference; *$p < 0.05$, **$p < 0.01$ vs. sham, #$p < 0.05$, ##$p < 0.01$ vs. vehicle-injected OVX group.

shown in Table 1, the OVX did not influence the concentration of serotonin, 5-HIAA, norepinephrine, or dopamine, in either tissue type. Administration of elcatonin to the OVX rats for 6 weeks also did not affect the monoamine levels, except for the concentration of 5-HIAA, which was significantly decreased in both tissue types, although the margins of decrease were small (16 and 12% in the spinal cord and the cerebrum, respectively), compared to the vehicle-treated OVX rats.

As expected, injection of PCPA significantly reduced the concentration of serotonin ($p < 0.01$) and 5-HIAA ($p < 0.01$) in both tissue types, but not that of other monoamines in the cerebrum, compared to the PCPA nontreated groups. In the spinal cord, the dopamine level, besides serotonin and 5-HIAA, decreased significantly ($p < 0.01$, Table 1) and the levels of 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid, metabolites of dopamine, also decreased (data not shown). The margins of decrease in the spinal cord of serotonin, 5-HIAA, and dopamine were 85, 89, and 19%, respectively, compared to the elcatonin-treated OVX group. These results suggest that the diminution of monoamines in the central nervous system by PCPA may have contributed to the complete abolishment of elcatonin-induced antinociception.

DISCUSSION

Besides inducing osteoporosis, we concluded that OVX induces hyperalgesia in rats, which in these experiments lasted over 7 weeks, even though there was no apparent alteration in the tail-withdrawal latency of the OVX rats following surgery, which is in contrast to Forman's observations (12) in which the tail withdrawal latencies decreased after OVX. The basis for our conclusion is that significant differences were observed in the latencies between the OVX group and the sham, and that the evaluation of bone change following OVX is conducted between the OVX group and the sham, and not between pre and post-OVX measurements (52). Further support for carrying out the comparison between the OVX group and the sham and not between pre- and post-OVX measurements may be seen in the gradual increase in tail-withdrawal latencies of the sham group over time, although the reason was unclear. This gradual increase may be related to the age of the rat, disallowing a comparison of pre- to post-OVX data. The weight of the rats used in this experiment and Forman et al. was 180–200 g and 225–250 g (12), respectively, which suggests the age of the two groups of rats was different, and hence, the response to OVX was different for each group. Experiments to reconfirm the occurrence of OVX-induced hyperalgesia are now being conducted with other nociceptive models, i.e., the acetic acid writhing and formalin models.

In clinical treatment with calcitonin for the pain accompanying osteoporosis, repeated injections for a month are required (13,14). However, to date, almost all the experimental studies conducted have demonstrated the acute antinociceptive effect of centrally (8,18,20,26,39,45,54) and systemically injected (9,27) calcitonin. Using the animal model of postmenopausal osteoporosis in rats, we demonstrated that subcutaneously and chronically administered elcatonin alleviated OVX-induced hyperalgesia in a dose-dependent manner and that the antinociceptive effect was only significant on and after 3 or 4 weeks of treatment. These experimental results are very similar to the clinical effects in patients (13,14).

TABLE 1

CHANGES IN SEROTONIN, 5-HYDROXYINDOLEACETIC ACID (5-HIAA), NOREPINEPHRINE AND DOPAMINE CONCENTRATIONS IN SPINAL CORD AND CEREBRUM

Organ	Operation	Injection	Serotonin	5-HIAA	Norepinephrine	Dopamine
Spinal cord	sham	vehicle	10.28 ± 0.39	3.77 ± 0.12	2.88 ± 0.09	0.276 ± 0.014
	OVX	vehicle	10.60 ± 0.56	3.95 ± 0.12	2.84 ± 0.09	0.312 ± 0.020
	OVX	elcatonin 20 U	9.50 ± 0.23	3.32 ± 0.15‡	2.64 ± 0.10	0.280 ± 0.010
	OVX	elcatonin 20 U, PCPA	1.47 ± 0.13*	0.35 ± 0.01*	2.58 ± 0.16	0.227 ± 0.010*
Cerebrum	sham	vehicle	11.04 ± 0.84	4.48 ± 0.09	2.84 ± 0.10	11.21 ± 0.66
	OVX	vehicle	10.57 ± 0.90	4.55 ± 0.15	3.06 ± 0.23	10.99 ± 0.51
	OVX	elcatonin 20 U	11.13 ± 0.59	3.99 ± 0.11†	3.13 ± 0.11	11.02 ± 0.38
	OVX	elcatonin 20 U, PCPA	1.84 ± 0.38*	0.48 ± 0.10*	2.96 ± 0.04	11.19 ± 0.54

Left half of brain and lumbar spinal cord were removed from rats, and the respective tissue from two rats were pooled. Concentrations of monoamines and a metabolite were measured by HPLC after homogenization and extraction. Each value is given as ng/mg protein and represents the mean of six data ± SEM.

Significant difference; *$p < 0.01$ vs. elcatonin-treated OVX group, †$p < 0.05$, ‡$p < 0.01$ vs. vehicle-injected OVX group.

Umeno et al. (49) reported that repeated systemic injections of elcatonin for 7 days inhibited hyperalgesia induced by formalin, and the effect lasted at least 24 h. In this study, we demonstrated that the OVX-induced hyperalgesia was not inhibited by the treatment of elcatonin for 2 weeks. The difference in the time required for the expression of the effect between the two studies may be due to the difference in the animal models for nociception. The effective duration of elcatonin antinociception on OVX-induced hyperalgesia was at least a week (our unpublished, preliminary data).

When calcitonin is evaluated in animals as an osteoporotic drug, not as an analgesic, it is necessary to allow more than a month to see the results of its effects because of the slow progression of bone formation and repair. Indeed, the inhibitory effect of elcatonin (20 U/kg) on the reduction of bone mineral densities in OVX rats was not significant after 4 weeks of treatment, whereas the antinociception was significant on and after 3 weeks. These results suggest that the clinical analgesic effect of calcitonin may be independent from the improvement in bone metabolism (14).

With the PCPA treatments, the antinociceptive effect of elcatonin was completely inhibited and the serotonin and 5-HIAA content in the spinal cord and the cerebrum significantly decreased to less than 20% of the elcatonin-treated OVX group. A significant decrease in dopamine and its metabolite content in the spinal cord was also observed after the PCPA treatments, although the margin of decrease was very small compared to those of serotonin and 5-HIAA. The content of norepinephrine was not influenced by the PCPA, although we did not examine the influence on norepinephrine metabolites. These results suggest the likelihood of involvement of the serotonergic system in elcatonin antinociception, but the data do not rule out the participation of other monoaminergic systems, including the dopaminergic.

Intracardially injected calcitonin did not bind to the central regions, such as the hypothalamus, in which calcitonin binding sites are distributed densely [(50), our unpublished data]. Although this evidence suggests that systemically injected calcitonin may not be able to pass through the blood–brain barrier, there is evidence indicating the central effects of systemically administered calcitonin. Plasma levels of ACTH and β-endorphin in humans (15,25) and those of β-endorphin in rats (32) increased with intramuscularly or intraperitoneally injected calcitonin, respectively. The evidence that monoamine content in whole rat brains changed after intramuscular (36) or SC (46) injection of calcitonin also indicates that systemically injected calcitonin affects the central nervous system. Our results also suggest a functional role of the central system(s) in the mediation of the antinociception of subcutaneously injected elcatonin for OVX-induced hyperalgesia. It remains to be elucidated how systemically injected elcatonin acts on any of the central systems.

The content of 5-HIAA was reduced significantly both in the brain and in the spinal cord of the elcatonin-treated OVX rats, compared with that of the vehicle-injected OVX rats, suggesting a decrease of serotonin release in both tissues. This seems to be logically inconsistent with the previous conclusion that the serotonergic system is involved in the antinociceptive effect of elcatonin, because an increase in serotonin release is generally required for acute antinociception where the serotonergic system is involved. However, the antinociceptive effect of elcatonin in this study was observed after long-term administration of the drug, which may not allow conventional mechanism models to be applied in the determination of whether the serotonergic system is involved in antinociception.

There is a reason supporting such a presumption. Nakhla and Majumdar (36) have reported that the serotonin content in rat brain increased rapidly, decreased to a normal level, and gradually increased again after 12 h with an intramuscular injection of calcitonin. If these acute and delayed increases in serotonin content are repeated in the brain by each injection, long-term administration of calcitonin may induce alterations in the serotonergic system, such as in the amount of released serotonin, the serotonin metabolic rate, or the number of serotonergic receptors. We expect that an alteration occurs in some factor in the OVX rats, which reverses itself in the elcatonin-treated rats, causing hyperalgesia and antinociception, respectively. Although the change in 5-HIAA content, which is one of the possible alterations, was detected between the vehicle-treated and the elcatonin-treated OVX rats, the change may not be so important because it was not detected between the sham and the vehicle-treated OVX rat. We are now examining the possibility of an alteration in the number of spinal serotonergic receptors among sham, vehicle-treated OVX, and elcatonin-treated OVX rats.

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