Change of β-Endorphin Concentration in Rat Brain after Administration of Indomethacin or Carrageenan

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This study aimed to investigate the behavior of an endogenous β-endorphin (β-EP) in the brain after subcutaneous (s.c.) injection of carrageenan or intravenous (i.v.) injection of indomethacin (IDM). The carrageenan was injected into rat hind paw subcutaneously in order to evoke only a local nociceptive stimulus. The β-EP concentration in the brain region was determined by radioimmunoassay at designated sampling times after the injection. It was observed that the β-EP concentration in the midbrain declined from 2.8±0.3 at 1 h to 1.3±0.02 ng/mg protein at 9 h. After the s.c. injection of carrageenan, the β-EP concentrations in the midbrain were found to be closely related to the nociceptive sensitivity which was determined by the Randall–Selitto test. On the other hand, a significant elevation of the β-EP concentration was observed in the hypothalamus from 3 h until 5 h compared with that of control. IDM was injected into rats at doses of 2.9, 5.8 and 8.6 mg/kg via the femoral vein. After i.v. administration of IDM, the β-EP increased in the hypothalamus, medulla oblongata, and midbrain, depending on the doses used. The value of hypothalamic β-EP concentration was two times higher than that of carrageenan. We found that nociceptive stimuli and IDM brought a change in the β-EP concentration in the brain of rats.

Key words β-endorphin; indomethacin; carrageenan; rat

MATERIALS AND METHODS

Chemicals IDM was a kind gift from Taisho Pharmaceutical Co., Ltd. (Saitama, Japan). Rat β-EP was purchased from Bachem Fine Chemicals, Inc. (Bubendorf, Switzerland). Antiserum to β-EP was purchased from Chemicon International, Inc., (CA, U.S.A.). Antiserum cross-reacted 100% with camel or rat β-EP, 78% with human β-EP and less than 0.1% with human β-lipotropin (61–77), Met-enkephalin and Leu-enkephalin, according to product sheets. (3,4-Dihydroxyphenylalanine) β-endorphin (human) was purchased from Amersham Corporation, (Arlington Heights, IL, U.S.A.). All other reagents were commercially available and of analytical grade.

Animals and Surgery Female Wistar rats (Japan SLC, Inc., Shizuoka, Japan) were housed in stainless steel cages under conditions of controlled temperature maintained at 23±1°C and a humidity of 55±10% during the investigation. Rats weighing 170–190 g, aged 8 weeks, were used throughout all the experiments. Free access to food and water was allowed.

Under light anesthesia with ethyl ether, a polyethylene catheter (PE 50 i.d., 0.58 mm; o.d., 0.965 mm; Clay Adams, Parsippany, NJ, U.S.A.) was inserted into the right femoral vein. The cannula was routed subcutaneously in the dorsum and exteriorized on the dorsal side of the neck. The rats were left for more than 24 h after surgery to exclude the effect for anesthesia (designated as the sham group). This surgery was carried out in the morning between 10:00 and 11:00.

Both the operated rats and intact rats were sacrificed by decapitation. Brain and pituitary were collected on an ice-cold glass plate. The brain was dissected into five regions according to the method of Glowinski and Iversen.

The tissues were transferred immediately to a polypropylene tube containing 2 ml of 1 M acetic acid. The tissues were heated at 95°C for 15 min, then homogenized with a polytron homogenizer at 4°C. The homogenate was centrifuged.

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at 10000×g for 20 min at 4 °C. The supernatant was lyophilized and stored at −80 °C until conducting the assay for β-EP.

**Determination of Nociceptive Sensitivity in Rats after Administration of Carrageenin.** 5.0 ml of 1.0% carrageenin saline suspension (Piccin-A, Lot No. P-14, Zushi Chemical Laboratory, Inc., Kanagawa, Japan) or the only vehicle was injected into the plantar surface of the rat hindpaw. At the time points of 0, 1, 2, 4, 5, 5.5, 6, 7, 8 and 9 h after the injection of carrageenin, the nociceptive sensitivity (NS) for pressure on the inflamed paw was determined by the Randall–Selitto test using an Analgesic Metre Ugo Basile (Biological Research Apparatus, Varese, Italy) as described previously. The NS was measured as the amount of pressure in weight required to induce the flight reaction (struggle) when pressure was applied to the inflamed paw, and expressed as a percent of the control.

**Influence of Carrageenin on β-Endorphin Concentration in Rat Brain**  Five rats were treated with carrageenin and another five rats were treated with saline subcutaneously, as noted above. At a designated time point after injection of carrageenin, the rats were sacrificed by exsanguination. The brain and pituitary were removed and used for the subsequent study to determine β-EP concentration.

**Time Courses of β-Endorphin Concentration in Brain after Indomethacin Administration**  Indomethacin dissolved in a small amount of poloxymethylene (20) sorbitan monoluate. IDM solution or the only vehicle was injected into rats via the right femoral vein at 24 h after the operation, respectively. Seven rats were treated as IDM-treated groups at doses of 2.9, 5.8 and 8.6 mg/kg, and another seven rats were treated as vehicle-treated group as well. After the injection, the rats were allowed freedom of movement in a plastic cage. The rats were decapitated at the time points of 10, 30, 60, 120 and 240 min after i.v. administration. Their brains and pituitaries were rapidly removed. The β-EP was extracted in acetic acid from the brain and the pituitary, as noted above. The β-EP concentration in lyophilized samples was determined by radioimmunoassay.

**Radioimmunoassay of β-Endorphin**  The slightly modified method of Tejwani was used as a radioimmunoassay for β-EP, as follows. Lyophilized tissue extracts were reconstituted in 50 mM phosphate buffer (pH 6.0) containing 50 mM NaCl, 5 mM EDTA, 0.1% gelatin, 0.1% Triton X-100 and 0.025% thimerosal. Samples of β-EP standards (0.1—100 ng in assay tube) were incubated for 24 h at 4 °C in polypropylene tubes with 100 μl of antibody diluted in an assay buffer. This incubation was followed by the addition of 100 μl of [125I] β-EP (about 20000 cpm per tube), and this was continued for an additional 24 h. Free and antibody-bound β-EP were separated by centrifugation after adding 0.5 ml of assay buffer containing 1.6% charcoal and 0.16% dextran T70. The radioactivity in the supernatant was determined in a gamma counter for 5 min. The concentrations of β-EP in the tissue were expressed as ng/mg protein. Concentrations of protein in the tissue samples were determined using the method of Lowry, with bovine serum albumin as a standard.

**Statistical Analysis**  Statistical analysis of the results was performed by analysis of variance (ANOVA) followed by Dunnett's multiple range test for multiple comparisons.

**RESULTS**

**Contents of β-Endorphin in Rat Brain**  A recovery experiment was carried out as follows. Rats were sacrificed by decapitation. Cerebella were transferred into polypropylene tubes containing 2 ml of 1 m acetic acid. The cerebella were heated at 95 °C for 15 min. After 30 min incubation on ice, 0.01, 0.1 and 1 ng of β-EP were added into the tube. The cerebella were homogenized and centrifuged at 20000×g for 20 min. Supernatants were assayed for β-EP by radioimmunoassay.

90.67±2.64, 91.66±1.87 and 93.18±6.15% of β-EP were recovered from 1 m acetic acid, respectively (0.01, 0.1 and 1 ng/tube of β-EP). Moreover, in the case of adding IDM to 1 m acetic acid, IDM did not affect the recovery of β-EP from the 1 m acetic acid.

Table 1 shows the brain concentration of β-EP in the intact rats and the sham rats. Among the tissues examined, the pituitary showed the highest concentration of β-EP (control: 1196.79±121.16 ng/mg protein, sham: 1182.13±69.30 ng/mg protein). Of all the brain regions, the highest concentration of β-EP was found in the hypothalamus, and the lowest was found in the midbrain. There was no significant difference in the concentrations in all regions between the intact rats and the sham rats.

**Time Courses of Nociceptive Sensitivity after Injection of Carrageenin**  Figure 1 shows the time courses of NS on the inflamed paw after the s.c. administration of carrageenin.

The NS dropped from 100.0±5.64% at time 0 to 41.3±3.27% at 4 h after the injection. The volume of edema was determined using the method of Arman et al. (data not shown). The volume rose from 0 to 72% for 6 h after the injection of carrageenin and was prolonged during the experimental period. With an increase in the volume of the edema,

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<th>Table 1. The Concentrations of β-Endorphin in the Brain of Rats</th>
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All values represent the means±S.E. of 5 animals, and are expressed as ng/mg protein.

**Fig. 1. Nociceptive Sensitivity after s.c. Injection of Carrageenin in Rats**

The nociceptive sensitivity was measured at designated times after the s.c. injection of carrageenin. All bars represent the means±S.E.M. of 5 animals.
Fig. 2. Time Courses of Endogenous $\beta$-Endorphin after s.c. Injection of Carrageen into Rats

The symbols (●, ■) represent the s.c. administration of carrageenin and physiological saline in the rat hind paw, respectively. All data represent the mean±S.E. of 5 animals. * p<0.05 vs. saline-treated rats, ** p<0.01 vs. saline-treated rats.

Fig. 3. Time Courses of $\beta$-Endorphin Induced in Brain of Rats after i.v. Administration of Indomethacin

Each point with vertical bars represents the means±S.E. of 7 animals. The symbols (●, △, ◆) represent 2.9, 5.8, 8.6 mg/kg of administration of IDM, respectively. The symbol (■) represents the administration of the vehicle as the control. * p<0.05 vs. sham-operated rats, ** p<0.01 vs. sham-operated rats.
the NS decreased.

**Influence of Carrageenin on β-Endorphin Concentration in Rats Brain**  Figure 2 shows the time course of β-EP concentration in the brain after the s.c. injection of carrageenin or saline. The β-EP concentrations changed in both the hypothalamus and midbrain after the injection. A significant elevation in β-EP concentration was observed in the hypothalamus from 3 h until 5 h compared with that of control. On the other hand, the β-EP concentration in the midbrain started diminishing at the time point of 5 h after the injection, and its value was maintained until the final sampling point, as shown in Fig. 2.

**Time Courses of β-Endorphin Induced by Indomethacin**  Figure 3 shows the time courses of β-EP concentrations in the brain after IDM or vehicle only administration. No ethopharmacological reaction against pain was observed in the rats when IDM or the vehicle was injected. An increase in β-EP concentration was observed in the medulla oblongata, hypothalamus and midbrain. In the hypothalamus, an increase was observed from 60 min until 240 min at three different doses. IDM produced a dose-related induction of β-EP in the hypothalamus and the medulla oblongata at the time point of 120 min after the injection of IDM. The maximum values (142±13.5 and 140±13.0 ng/mg protein) of hypothalamic β-EP concentration at doses of 5.8 and 8.6 mg/kg were about four times higher compared with the vehicle-treated rats. These values corresponded to the reported value59 (about 150 ng/mg protein) of the concentration of β-EP induced by diclofenac. Also, it was clearly observed that the β-EP concentration in the midbrain increased depending on the doses during the experimental term.

However, although the β-EP concentration decreased in the pituitary significantly following the injection of IDM, a dose-related decline in β-EP was hardly observed in the pituitary.

**DISCUSSION**

It has been reported that some NSAIDs affect the mechanisms of analgesia in the central nervous system.6,18–30 There is direct evidence that β-EP was induced by some NSAIDs in these reports.6,18 Our data also indicate that β-EP concentration in the rat brain. It is interesting that endogenous β-EP in the brain was induced by IDM, which inhibits PG in the peripheral tissues.

Dolores et al.31 reported that ether has an ability to increase β-EP concentration in the rat pituitary. In their experiments, the concentration in the pituitary increased more than 5 times compared with intact rats at 30 min after ether exposure. Therefore, our rats were maintained for 24 h after the operation in order to exclude the influence of ether in our experiments. The concentrations of β-EP in the pituitary were reduced to basal level (about 1200 ng/mg protein) at 24 h after the operation, as shown in Table 1. This result supports a variation of β-EP concentration being due to IDM or carrageenin administration.

It was observed that β-EP concentration is elevated in the hypothalamus and declines in the midbrain after a s.c. injection of carrageenin, as shown in Fig. 2. However, in the pituitary and the medulla oblongata, there was no significant difference in the β-EP concentration between carrageenin-treated rats and saline-treated rats. These results indicate that the regions can be classified into two groups. One group consists of the hypothalamus and midbrain, the other group consists of the pituitary and medulla oblongata. The nociceptive stimuli which are caused by carrageenin were transmitted to the lamina I and II of the dorsal horn by an afferent C fiber, and reached from the spinal cord to the thalamus through a lateral spinothalamic system via the hypothalamus.32 The classification of the regions, thus, can be understood by means of considering the pathway of the nociceptive stimuli because the midbrain (including thalamus) and hypothalamus were located in this pathway. However, it is difficult to elucidate the result of the decline in the β-EP concentration in the midbrain. Therefore, we showed the relationship between the β-EP concentration in the midbrain and the NS in Fig. 4. After the s.c. injection of carrageenin, the β-EP concentrations in the midbrain were found to be closely related to the NS. In the previous report, we used the carrageenin in order to evaluate the analgesic activity of IDM. The β-EP that was induced by carrageenin hardly affected the evaluation. The reason for this is that steady state β-EP concentration was observed during 5 to 9 h after the s.c. injection of carrageenin (Fig. 2), and the evaluated analgesic activity was expressed as a difference between the analgesic activity in IDM-treated rats and that in vehicle-treated rats at each time point after the injection.5 In Fig. 3, the hypothalamus and pituitary are considered to be interesting regions regarded as locations of the induction of β-EP by IDM because the β-EP in these regions was found in higher concentrations than in the other regions. As is already well known, the hypothalamus is the most important site of β-EP synthesis in the brain and is involved in several important functions such as mood, endocrine regulation and pain.10,11 A maximum value of β-EP concentration in the hypothalamus after the i.v. administration of IDM corresponded to the maximum value of β-EP concentration induced by diclofenac.10 This correspondence suggests that IDM possesses the ability to induce β-EP in the hypothalamus as well as diclofenac does. IDM and diclofenac are classified into a group of aryl acetic acid based on their chemical
structure. It is known that the drugs of the aryl acetic acid group have the strongest analgesic effect among NSAIDs. This strong analgesic effect of aryl acetic acid may result from the induction of β-EP in the hypothalamus.

We consider that the induction of β-EP is involved in the analgesic effect of IDM. Our hypothesis obtained support from the results that β-EP concentrations increased in the medulla oblongata and the midbrain for the following reason. The nucleus reticularis gigantocellularis (NRGC) and periaqueductal gray matter (PAG) are located in the medulla oblongata and midbrain, respectively. The descending inhibitory system, which played the role of inhibiting the impulse of pain, started from these sites to the spinal cord. In particular, PAG was considered the site of action of β-EP. Moreover, clonipramine induced analgesia and β-EP in the hypothalamus, simultaneously, despite being a tricyclic antidepressant drug. In that report, the value of hypothalamic β-EP concentration was about 100 ng/mg protein at 30 min after clonipramine administration. In our experiments, the value of β-EP concentration in the hypothalamus was sufficient to elicit analgesia because its value exceeded 100 ng/mg protein at doses of 5.8 and 8.6 mg/kg of IDM.

It has been reported that the pituitary is directly connected to the hypophysial vein. Because circulating blood flows into the pituitary, there is a morphic difference between the pituitary and the brain. After the i.v. administration of IDM, the pituitary was the only region in which β-EP concentration decreased. This result might be a reflection of such a morphic difference.

Although IDM could not penetrate across the blood–brain barrier in the rat after i.v. administration, IDM taken in an overdose induces toxicity in the central nervous system, such as headache, tinnitus, dizziness, lethargy, drowsiness, confusion, disorientation and restlessness. This fact suggests that IDM affects the central nervous system through several pathways. In addition, β-EP exerted an analgesic effect resulting from the injection of β-EP into the rat brain. Therefore, we consider that β-EP contributed to part of the analgesic effect of IDM in rats. However, to determine the percentage of the analgesic of β-EP further experiments are necessary.

In conclusion, we clarified that β-EP was induced in rat brain by the injection of IDM and carrageenin. Carrageenin induces nociceptive stimuli in the peripheral tissues in order to determine the analgesic activity of NSAIDs. On the other hand, IDM reduces nociceptive stimuli. These drugs were used for evaluating the analgesia of IDM, simultaneously. Hence, the present data are important in the evaluation of the analgesia of IDM.

Acknowledgment This work was supported in part by a Grant-in-Aid for Scientific Research (07307035) from the Ministry of Education, Science, Sports and Culture, Japan.

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