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# Study of the Antinociception Induced by Opioids and the Proenkephalin Gene Expression in Spontaneously Hypertensive Rats

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## ABSTRACT

The present studies were carried out to determine if antinociceptive action of morphine and β-endorphin administered intraventricularly was changed in pentobarbital anesthetized spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Antinociception was assessed by the tail-flick test. The $ED_{50}$ values of antinociception for morphine administered intraventricularly were 1.9 and 1.2 nmol for WKY and SHR rats, respectively. The $ED_{50}$ values of antinociception for β-endorphin administered intraventricularly were 0.40 and 0.12 nmol for WKY and SHR rats, respectively. The [$Met^5$]-enkephalin (ME) and proenkephalin mRNA levels in midbrain, pons and medulla, or lumbar section of the spinal cord in WKY and SHR rats were measured by the radioimmunoassay and Northern blot assay, respectively. There were no differences of ME and proenkephalin mRNA levels in these tissues between WKY and SHR rats. The results suggest that β-endorphin but not morphine administered intraventricularly produces a greater antinociception in SHR rats. This increased antinociceptive effect of β-endorphin in SHR rats may be not, at least, due to the alterations of ME and proenkephalin mRNA levels in the midbrain, pons and medulla, or spinal cord.

**Key Words:** Morphine, β-endorphin, Antinociception, [$Met^5$]-enkephalin, Proenkephalin mRNA, Brain, Spinal cord

## INTRODUCTION

The antinociceptive effects induced by β-endorphin and morphine are mediated by the activation of different neural mechanism. We have previously proposed that the antinociception induced by β-endorphin and morphine given intracerebroventricularly is mediated by stimulating specific ε-and μ-opioid receptors, respectively (Suh and Tseng, 1988, 1990, a and b; Suh *et al.*, 1988, 1989). This hypothesis is based on the findings that β-endorphin (1-27) administered intracerebroventricularly selectively antagonizes antinociception induced by β-endorphin but not morphine injected intracerebroventricularly (Suh *et al.*, 1988). β-Funaltrexamine, CTOP (D-Phe-Cys-Tyr-D-Tyr-Orn-Thr-Pen-Thr-$NH_2$, or naloxone administered intracerebroventricularly effectively antagonizes antinociception induced by mor-

phine, but not β-endorphin (Suh and Tseng, 1988, 1990a; Suh *et al.*, 1989). Furthermore, one intracerebroventricular injection of morphine or β-endorphin induces acute antinociceptive tolerance to its own distinctive opioid receptor and does not cross-tolerance to other opioid agonist with different opioid receptor specificities (Suh and Tseng, 1990b).

Recent studies have demonstrated that the antinociceptive effect induced by morphine or other μ-opioid receptor agonists administered systemically or supraspinally was equal or greater in SHR rats (Bhargava, 1982; Widy-Tyszkiewicz and Czlonkowski, 1989) while supraspinally administered β-endorphin-induced antinociception is SHR and WKY rats has not been characterized. Since several evidence have demonstrated that the antinociceptive action of β-endorphin administered supraspinally is mediated by different system from that of morphine, it is expected that the antinociceptive effects of β-endorphin and morphine administered supraspinally may be different between SHR and WKY rats. Therefore, the present study was designed to characterize the antinociception induced by β-endorphin and morphine administered intraventricularly in SHR and WKY rats. In addition, to reveal the mechanisms involved in opioid-induced response in SHR rats, [Met$^5$]-enkephalin (ME) and proenkephalin mRNA levels were examined in midbrain, pons and medulla, or spinal cord, which are important regions for the production of antinociception by opioids.

## MATERIALS AND METHODS

### Subjects

Male SHR and WKY rats were used. Animals were housed 2 per group in a room maintained at 22±0.5℃ with an alternating 12-hr light-dark cycle. Food and water were available ad libitum. Each animal was used only once.

### Determination of blood pressure

Before estimation of the antinociceptive activity of opioids, systolic blood pressure of both strains of rats was measured between 9:00-12:00 AM using a tail-cuff method.

### Antinociception test

The experiments were carried out in pentobarbital-anesthetized rats. Rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) and were mounted in a stereotaxic apparatus (David Korf Instruments, Tujunga, CA). Pentobarbital but not urethane was used as an anesthetic agent in the tail-flick test because urethane at an anesthetic dose inhibits the tail-flick response. The anesthetic state was maintained by injecting 6 to 10 mg/kg of pentobarbital sodium every 45 min or as needed. An injection cannula made of 30-gauge stainless-steel tubing connected to a 10 μl Hamilton microsyringe with a segment of PE-10 tubing was prefilled with morphine or β-endorphin solution and were inserted stereotaxically into the 3rd ventricle. A Cummulative dosing schedule was used for morphine and β-endorphin. Morphine and β-endorphin at the dose of 0.5 and 0.25 μg, respectively, for the first injection and doubling the doses for subsequent injection, was injected into the 3rd ventricle every 20 min. The antinociceptive response induced by morphine were assessed by the tail-flick test (D'Amour and Smith, 1941; Dewey and Harris, 1975). The intensity of the radiant heat for the tail-flick response was adjusted so that the animal flicked the tail in 4~6 sec before intraventricular injection of morphine or β-endorphin. The tail-flick response was measured before and 20 min after intraventricular injection. Changes of latency of the tail-flick responses were expressed as percentage antinociception, which was calculated $[T_1-T_0)/(T_2-T_0)] \times 100$, where $T_0$ and $T_1$ were the tail-flick latencies before and after the injection of opioid agonist and $T_2$ was the cutoff time which was set at 10 sec for the tail-flick test.

### Radioimmunoassay

The level of ME-like immunoreactivity was determined by radioimmunoassay (RIA) as described (Hong *et al.*, 1978). After decapitation, brain regions and lumbar section of the spinal cord of the rats were immediately diffested and frozed on dry-ice. Tissue was homogenized in 2M acetic acid at 4℃. Homogenates were heated in boiling water for 5 min and centrifuged at 15,000 × g for 20 min. Supernatant were lyophilized to

dryness. The residues were reconstituted in RIA buffer and incubated with $^{125}$IME. The ME level was determined in duplicate for each sample. Iodinated ME (10,000 cpm) was incubated overnight at 4℃ with various concentrations of ME as the standard or extracts from tissues, and the rabbit antiserum against ME in a final volume of 0.5 ml. Separation of bound ME from free ME was accomplished by incubation with 0.2 ml of a charcoal slurry containing 1.35% bovine serum albumin (BSA) in RIA solution for 20 min followed by centrifugation at 4,000 × g for 10 min. Five hundred $\mu$l of the supernatant was counted for radioactivity. The antiserum to ME was raised by immunization of rabbits with ME conjugated to thyroglobulin antigen and had the following cross reactivities to the opioid peptides: ME, 100%; [Met-O$^5$] enkephalin, 80%; [Met$^5$] enkephalin-Arg$^6$-Phe$^7$, <0.4%; [Leu$^5$]-enkephalin, <1%; dynorphin (1-8), <0.06%; and $\beta$-endorphin, <0.2%.

**Isolation of RNA and Northern blot analysis**

Total RNA was extracted from brain and spinal cord tissues in a single step by a guanidinium isothiocyanate/phenol/chloroform gradient procedure (Chomczynski and Sacchi, 1987) and the cellular RNA in the aqueous phase was precipitated with an equal volume of ice-cold isopropanol. The concentration of RNA was determined spectrophotometrically at 260 nm. The relative abundance of proENK mRNA was assayed by Northern blot analysis as described (Chomczynski and Sacchi, 1987). Ten $\mu$g of RNA were denatured and electrophoresed on a 1.2% agarose gel and then transferred to a nylon hybond-N hybridization filter sheet (Amersham Co., Arlington Heights, IL). After baking for 2 hr at 80℃, transfer membranes were prehybridized in a buffer for at least 4 hr at 42℃. The radiolabeled proENK probe (5′-GCC GAG CGC CAG CAG CCA AGT GCA GAG TCC CAG GAA CCG CGC-3′) was added at a specific activity of 1 × 10$^7$ cpm/ml and the membrane was incubated overnight (>14 hr) at 42℃. Following hybridization, the membranes were washed three times in 2X SSC (1X= 0.015 M sodium citrate and 0.15 M NaCl) containing 0.1% SDS (sodium dodecyl sulfate) at 42℃ for 20 min. The membranes were then dried and exposed to Kodak XAR-5 film for 1~3 days at −70℃.

**Statistical analysis**

A unpaired Student's t-test was used to compare the contents of ME in various regions of the brain and spinal cord. In antinociception study, the median effective dose ($ED_{50}$) and 95% confidence intervals were determined by the method of Litchfield and Wilcoxon (1949) with the aid of a computer program described by Tallarida and Murray (1981).

**Drugs used**

The drugs used were $\beta$-endorphin (Peninnsula Laboratories, Ind., Belmont, CA), morphine HCl (Sam-Sung Pharmaceutical Comp., Seoul., Korea). All drugs used were dissolved in a sterile saline (0.9% NaCl).

## RESULTS

**Effect of morphine and $\beta$-endorphin administered intraventricularly on inhibition of the tail-flick response in WKY and SHR rats**

Table 1 gives mean values for body weights and systolic blood pressures for SHR and WKY rats aged 8, 18 and 32 weeks. Arterial pressure increased gradually with age in each of these

**Table 1.** Body weights and blood pressure of rats of *various ages*

| Age, weeks | SHR | WKY |
|---|---|---|
| Body weight, g. | | |
| 8 | 185.3±10.5 | 185.5± 8.3 |
| 18 | 276.0±10.2 | 378.0±12.7 |
| 32 | 303.8±11.3 | 385.0± 8.2 |
| Systolic blood pressure, mmHg | | |
| 8 | 149.0± 4.4 | 120.0± 2.3 |
| 18 | 190.0± 9.5 | 138.0± 6.4 |
| 32 | 196.0± 5.0 | 128.0± 5.4 |

Male SHR and their normotensive control WKY rats, 8, 18, and 32 weeks old, were used in this study. The arterial systolic pressure was measured in conscious restrained rats between 9:00~12:00 AM, 3 days prior to sacrifice, using a tail-cuff method. Values listed above are means±SEM of 9~10 rats.

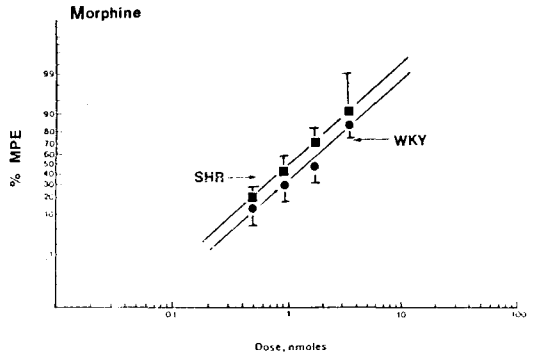


Fig. 1. Effect of morphine administered intraventricularly (i. vt.) on the inhibition of the tail-flick response. Morphine at increasing doses (0.5~8 μg) was injected into the 3rd ventricle every 20 min. The inhibition of the tail-flick response was measured before and after each intraventricular injection of morphine. % MPE denotes the maximal possible effect. The vertical bars indicate the standard error of the mean. The number of animals used for each curve was 6.

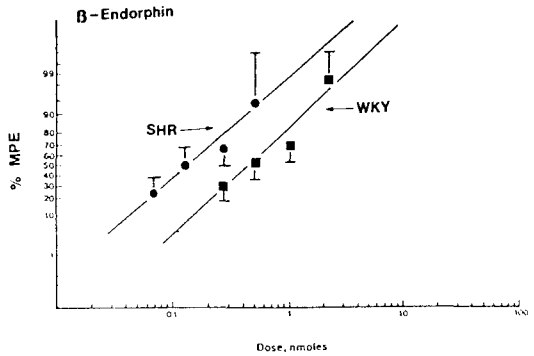


Fig. 2. Effect of β-endorphin administered intraventricularly (i. vt) on the inhibition of the tail-flick response. β-Endorphin at increasing doses (0.25~8 μg) was injected into the 3rd ventricle every 20 min. The inhibition of the tail-flick response was measured before and after each intraventricular injection of β-endorphin. % MPE denotes the maximal possible effect. The vertical bars indicate the standard error of the mean. The number of animal used was 6.

**Table 2.** Effect of intraventricular injection of morphine or β-endorphin on inhibition of the tail-flick response in SHR and WKY rats

| Drug | $ED_{50}$(nmol/rat)[a] | |
|---|---|---|
| | SHR | WKY |
| Morphine | 1.20 (0.62~0.21)[b] | 1.90 (0.81~2.91) |
| β-endorphin | 0.12[c] (0.05~0.21) | 0.40 (0.23~0.78) |

[a], $ED_{50}$ values were calculated according to the method described by Litchfield and Wilcoxon (1949).

[b], Numbers in the parentheses indicate the 95% confidence interval.

[c], Significantly different from WHY rats at $P<0.05$.

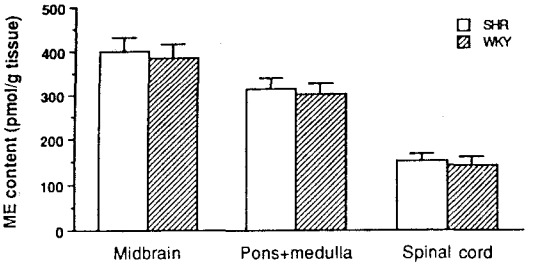


Fig. 3. The level of [Met[5]]-enkephalin (ME)-like immunoreactivity in midbrain, pons-medulla, and the lumbar section of the spinal cord in SHR and WKY rats. ME levels were measured by radioimmunoassay. The vertical bars indicate the standard error of the means. n=9-10 rat brains or spinal cords.

groups, with the mean levels of SHR being slightly higher than normotensive WKY rats at 8 weeks of age and significantly higher at 18 and 32 weeks of age.

The antinociceptive effects of morphine and β-endorphin were tested in 18-week-old SHR and WKY rats. As shown in Fig. 1 and 2, intraventricular injection of morphine or β-endorphin caused a dose-related increase of inhibition of the tail-flick response in WKY rats. The dose-response line for antinociception induced by intraventricular injection of morphine were shifted to the left

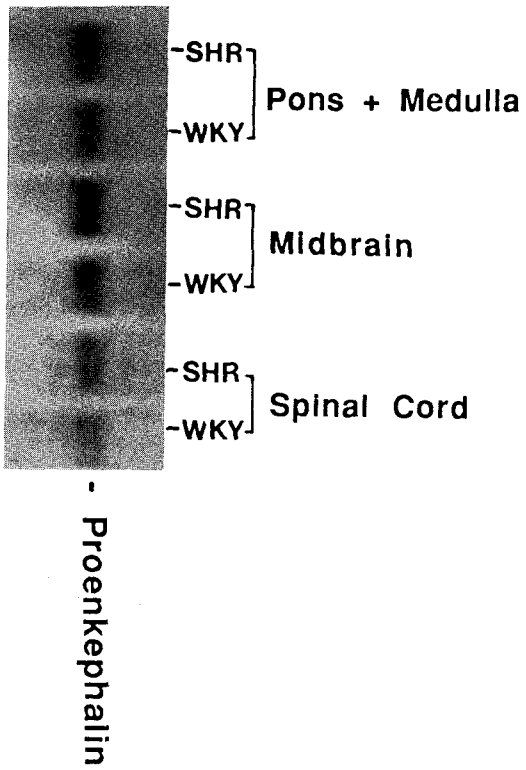


**Fig. 4.** The proenkephalin A mRNA levels in midbrain, pons-medulla, and the lumbar section of the spinal cord in SHR and WKY rats. Ten $\mu$g of total RNA were used for the determination of proenkephalin mRNA levels by a Northern blot assay, as described under "Materials and Methods".

slightly (Fig. 1, Table 2). However, the dose-response line for antinociception induced by intraventricular injection of $\beta$-endorphin were significantly shifted to the left, without altering the curve of the dose-response line (Fig. 1, Table 2).

**ME and proenkephalin A mRNA levels in midbrain, pons and medulla, or spinal cord**

Fig. 3 presents mean data for ME-like immunoreactivity in midbrain, pons and medulla, or spinal cord of 18-week-old SHR and WKY rats, when hypertension is fully developed in SHR. None of the differences between SHR and WKY brains and the spinal cord were statistically significant. Similarly, there were no differences in proenkephalin A mRNA levels in midbrain, pons and medulla, or spinal crod between SHR and WKY rats(Fig. 4).

## DISCUSSION

The results of the present studies demonstrate that $\beta$-endorphin administered intraventricularly produces a greater antinociceptive effect in SHR than that in WKY rats. However, the antinociceptive effect of morphine administered intraventricularly was slightly, but not significantly, greater in SHR than that in WKY rats. The results support our previous hypothesis that the antinociceptive actions of morphine and $\beta$-endorphin administered supraspinally are mediated by different neuronal mechanisms.

Our previous findings have suggested that $\beta$-endorphin administered supraspinally produces antinociception by releasing ME from the spinal cord. The midbrain, brainstem and the spinal cord are important regions for the regulation of antinociception by opioids. These regions contains enkephalin inter-neurons and enkephalin neurons project to the spinal cord. Therefore, we examined the level of ME and proenkephalin A mRNA levels in these regions and found that no differences of ME and proenkephalin A mRNA levels in midbrain, medulla and pons, and the spinal cord between SHR and WKY rats, suggesting that the enhancement of antinociception induced by $\beta$-endorphin in SHR was not due to the changes of ME and proenkephalin biosynthesis in regions of the brain and the spinal cord. Our finding on ME level in SHR and WKY rats were in line with those reported by several other groups (Bhargava *et al.*, 1988; Li *et al.*, 1992). However, Hoegler *et al* (1989) have reported that, at 12-weeks, proenkephalin A mRNA levels were increased in midbrain and spinal cord of SHR relative WKY rats, while proenkephalin mRNA level was significantly reduced in the pons-medulla of SHR relative to WKY rats. These differences are not clear now.

In order studies, it was found that the basal concentration of $\beta$-endorphin in plasma was similar in SHR and WKY rats (Bucher *et al.*, 1987). $\beta$-Endorphin cell bodies are primarily located in ar-

cuate nucelus in hypothalamus. These endorphinergic neurons project to the midbrain and brainstem regions. The contents of β-endorphin in the hypothalamus, midbrain, and brainstem were also similar in SHR and WKY rats (Gaida *et al.*, 1984; Bharagava *et al.*, 1988). To delineate the antinociceptive mechanisms of β-endorphin, further studies, such as a biochemical β-endorphin receptor binding study and releasibility of ME from the spinal cord by supraspinally administered β-endorphin, need to be investigated.

In contrast to the action of β-endorphin, the antinociceptive effect induced by morphine administered intraventricularly was slightly, but not significantly, enhanced in SHR. This result was in part in line with finding by Widy-Tyszkiewicz and Czlonkowski (1989) in that they have shown that the antinociceptive effect of morphiceptin, a μ agonist, was not differ between both strains of the rats. However, Bharagava (1982) has reported that a greater antinociceptive response to systemically injected morphine was observed in SHR than in WKY rats. We have previously reported that morphine produces antinociception by stimulating μ-opioid receptors at supraspinal level (Suh and Tseng, 1988, 1990, a and b; Suh *et al.*, 1988, 1989). Radioligand binding studies have recently shown that the $K_d$ and $B_{max}$ values for opioid receptors in midbrain, pons-medulla, and spinal cord were not altered when naltrexone was used as a radioligand (Rahmani *et al.*, 1991). However, when [$^3$DAMGO], a specific μ-opioid receptor agonist, was used as a ligand, $B_{max}$ value was increased in midbrain but not in ponsmedulla and spinal cord, without altering the $K_d$ value in these regions (Gulati, 1990). Furthermore, there was no change in the μ-receptor binding activity in the central gray matter regions (Kujirai *et al.*, 1991). In the present study, the slight enhancement of antinociceptive effect induced by morphine administered intraventricularly in SHR might be due to the increased number of μ-opioid receptors in the midbrain region.

We and other have demonstrated that morphine administered supraspinally produces antinociception by releasing norepinephrine or serotonin from the spinal cord (Kuraishi *et al.*, 1978, 1979; 1983; Jung *et al.*, 1994). Between 8 and 16 weeks, increased norepinephrine levels were observed in the nucleus reticularis gigantocellularis, which is a region sensitive to morphine for producing antinociception, of SHR compared with WKY rats (yao *et al.*, 1989). It would be interesting to examine whether microinjection of morphine into the nucleus reticularis gigantocellularis causes the release of greater amount of norepinephrine in SHR.

Evidence have been accumulated that β-endorphin and morphine stimulate different types of opioid receptors for antinociception at supraspinal sites. β-Funaltrexamine and naloxone, selective mu-opioid receptor antagonists, given intracerebroventricularly effectively antagonize the antinociception induced by morphine administered intracerebroventricularly but are not effective or less effective in antagonizing the antinociception induced by β-endorphin administered intracerebroventricularly (Suh and Tseng, 1988; Suh *et al.*, 1989). β-Endorphin-(1-27) injected intracerebroventricularly antagonizes the antinociception induced by β-endorphin administered intracerebroventricularly but does not affect the antinociceptive effects of morphine (Suh *et al.*, 1988; Hammonds *et al.*, 1984; Nicholas and Li, 1985), suggesting that the differential effects of morphine and β-endorphin administered intraventricularly on the inhibition of the tail-flick response in SHR and WKY rats may be attributed to the stimulation of different opioid receptors at supraspinal sites; μ-opioid receptors for morphine and ε-receptors for β-endorphin.

Other studies have further demonstrated that β-endorphin and morphine produce their antinociception by activating separate descending systems. We have previously demonstrated that β-endorphin administered supraspinally causes a release of immunoreactive Met-enkephalin from the spinal cord in urethane anesthetized rats (Tseng *et al.*, 1985, 1986, a and b; Suh and Tseng, 1990c, 1992a). In addition, we found that pretreatment of mice intrathecally with thiorphan and bestatin effectively potentiated inhibition of the tail-flick response induced by β-endorphin administered intracerebroventricularly (Suh and Tseng, 1990, c and d). Furthermore, we have recently found that thiorphan and bestatin added into the perfusate for intrathecal perfusion increased markedly the Met-enkephalin content in the spinal perfusate released by β-endorphin in-

jected supraspinally (Suh and Tseng, 1990c). The involvement of Met-enkephalin in β-endorphin-induced antinociception is also supported by other studies. ① Blockade of opioid receptors by intrathecal injection of naloxone or other opioid antagonists effectively antagonizes inhibition of the tail-flick response induced by β-endorphin given supraspinally (Tseng and Fujimoto, 1984, 1985; Suh and Tseng, 1988; suh *et al.*, 1989). ② A single intracerebroventricular injection of β-endorphin produces an acute tolerance to δ-opioid receptor activity for antinociception in the spinal cord (Suh and Tseng, 1990b). ③ Intrathecal injection of antibody to Met-enkephalin but not antibodies to Leu-enkephalin, dynorphins and β-endorphin antagonizes inhibition of the tail-flick response induced by β-endorphin given intracerebroventricularly in mice (Tseng and Suh, 1989). ④ Intrathecal injection of Met-enkephalin and other opioids produce antinociception (Schmauss and Yaksh, 1984; Yaksh, 1983). The evidences described above give support to the contention that Met-enkephalin is the mediator for β-endorphin-induced antinociception.

We have previously found that ① morphine given intracerebroventricularly does not release Met-enkephalin from the spinal cord (Tseng *et al.*, 1985). ② Intrathecal injection of naloxone or other opioid receptor blockers does not antagonize inhibtion of the tail-flick response induced by morphine given intracerebroventricularly (Tseng and Fujimoto, 1984; Suh and Tseng, 1988; Suh *et al.*, 1989). ③ Intrathecal injection of antibody to Met-enkephalin does not antagonize inhibition of the tail-flick response induced by morphine given intracerebroventricularly (Tseng and Suh, 1989). ④ inhibition of the degradation of Met-enkephalin by intrathecal injection of thiorphan or bestatin did not potentiate inhibition of the tail-flick response induced by morphine given intracerebroventricularly (Suh and Tseng, 1990c). These informations indicate that Met-enkephalin and its associated opioid receptors in the spinal cord are not involved in supraspinally administered morphine-induced inhibition of the tail-flick response.

Several lines of evidence have further dimonstrated that β-endorphin and morphine procuce their antinociception by activating separate descending systems. ① Blockade of the spinal serotonergic receptors by methysergide effectively antagonized the inhibition of the tail-flick response in duced by morphine but not that induced by β-endorphin administered into periaquenductal gray, rostroventral medulla, or intracerebroventricularly (Suh *et al.*, 1989; Yaksh, 1978, 1979; Jensen and Yaksh, 1984, 1986; Kuraishi *et al.*, 1978, 1979, 1983; Wigdor and Wilcox, 1987). ② The inhibition of serotonin reuptake by fluoxetine selectively potentiated inhibtion of the tail-flick response induced by morphine but not that induced by β-endorphin administered intracerebroventricularly (Suh and Tseng, 1990, c and d). ③ Intrathecal injection of serotonin produce antinociception (Reddy *et al.*, 1980; Yaksh and Wilson, 1979; Howe *et al.*, 1983). ④ Depletion of serotonin in the spinal cord by intrathecal injection of a neurotoxic drug, 5,7-dihydroxytryptamine, antagonizes the antinociceptive effects of morphine administered intracerebroventricularly or systemically (Suh *et al.*, 1992b; Sawynok and Reid, 1987; Mohrland and Gebhart, 1980). However, degeneration of serotonergic neurons produced by 5,7-dihydroxytryptamine was ineffective in altering the inhibition of the tail-flick response induced by intracerebroventricularly administered β-endorphin (Suh *et al.*, 1992b). The findings indicate that descending serotonergic pain inhibitory systems are involved in supraspinally administered morphine-induced antinociception.

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=국문초록=

# 선천성 고혈압쥐에서의 Opioid에 의한 진통작용과 Proenkephalin유전자 발현에 대한 연구

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8주, 18주, 그리고 32주된 Spontaneously Hypertensive Rats (SHR)과 Wistar-Kyoto Rats (WKY)과의 blood pressure (혈압)를 측정하여본 결과 SHR 그룹이 WKY에 비해 19에서 70 mmHg 차이로 SHR 그룹이 WKY group에 비하여 혈압이 높았다. 18주된 SHR과 WKY에서 제 3 뇌실내 (intraventricular)로 투여된 morphine과 β-endorphin의 진통작용을 검색하여 보았다. WKY group에 비하여 SHR group에서 뇌실내로 투여된 β-endorphin은 진통작용에 있어서 상승작용 (potentiation)을 보임을 발견하였고 뇌실내로 투여된 morphine은 SHR group에서 약간만 상승작용을 보였다. SHR과 WKY group간에 opioid의 진통작용에 있어서 중요한 역할을 하는 Midbrain과 Medulla (pons), 그리고 spinal cord (척수)의 lumbar부위의 [Met$^5$]-enkephalin과 proenkep-halin A mRNA level을 측정하여 보았다. SHR과 WKY group간의 [Met$^5$]-enkephalin과 proenkephalin mRNA의 양은 별로 차이를 보이지 않았다. 이러한 결과로 미루어 볼때 SHR group에서 뇌실내로 투여된 β-endorphin은 그의 진통효과에 있어서 보인 상승작용은 척수상부에 위치하고 있는 opioid deptide의 양이 변해서가 아니라 다른 기전에 의하여 조절되어지고 있음을 시사한다.