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Inhibitory Action of the Natural Product AP1700 on the Withdrawal Syndrome of Nalbuphine

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Abstract – The study was undertaken to determine the antagonism of the AP1700 on the development of nalbuphine-induced tolerance and physical dependence. AP1700 is an oriental drug preparation composed of 5 natural products and is known to have antinarcotic action with an oral dose of 250 mg/kg in rats. AP1700 significantly inhibits the development of nalbuphine-induced physical dependence but does not the tolerance. Mitogen-activated protein kinase, which include extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) play critical roles in cell growth and survival and drug abuse. The level of pCREB was elevated in the hippocampus by the chronic treatment with nalbuphine, however, the elevation of pCREB was not inhibited by the AP1700 co-treatment. Interestingly, the level of pERK was decreased in the co-treatment with nalbuphine and AP1700 on the cortex and striatum. However, the level of nNOS and NR1 was not modulated by the treatment with nalbuphine or AP1700 on the cortex, hippocampus and striatum in the rat brain. These results suggest that the AP1700 could be used to ameliorate the nalbuphine withdrawal symptoms.

Keywords ☐ analgesia, tolerance, dependence, glutathione, pERKIntroduction

The analgesic action of opioid is very remarkable. But repeated treatment with opioid produces physical dependence, characterized by withdrawal symptoms and a tolerance. Thus, there must be a continuing search for morphine-type compounds which are devoid of addiction liability and are orally effective antinarcotic agent or preparation with lesser side effects. The analgesic nalbuphine has an interesting pharmacological profile both in animals and in humans. Nalbuphine, an opioid mixed agonist-antagonist, is structurally related to the potent opioid, oxymorphone, and the potent opioid antagonist, naloxone (Walker and Young, 1993; Chen *et al.*, 1992). Nalbuphine analgesia has been classified as kappa, but its lower incidence of behavioral side effects distinguishes it from other mixed agonist/antagonist agents such as pentazocine (Schmidt *et al.*, 1985). The nalbuphine has a low dependence profile, possibly related to its ability to an antagonize morphine and other mu opioid drugs (Schmidt *et al.*, 1985). For the prevention of opioid-related side effects, both nalbuphine and naloxone can effectively decrease the incidence of respiratory depression, nausea, vomiting, and pruritus (Penning *et al*,. 1988).

The abuse potential of opioids involves not only their subjective effects and consequent self-administration, but also their ability to induce physical dependence. The dysphoric effects of withdrawal in the physically dependent individual are a strong stimulus to maintain compulsive drug-seeking behavior and self-administration. These facts were recognized early in the modern search for opiate analgesics of lowered abuse potential and led to the development of a number of animal models of opioid physical dependence. These assays rely on the detection of the signs and symptoms of abstinence or withdrawal in animals related chronically with the test compound. The rapid screening of compounds for physical dependence liability may be best done with mice and rats. After chronic exposure to mor-

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phine by means of morphine pellet implantation or multiple injections, mice or rats display abstinence syndrome characterized by stereotyped behavior after naloxone challenge treatment. It has been suggested that NMDA receptor and/or NO is involved in the penomena of opioid dependence and withdrawal (Oh *et al.*, 2000; Cuellar *et al.*, 2000). When non-competitive NMDA receptor antagonists such as ketamine, MK-801, and dextromethorphan are administred immediately prior to naloxone-precipitated morphine withdrawal, signs of withdrawal syndrome are attenuated (Koyuncuoglu *et al.* 1992; Tokuyama *et al.* 1996). The physiological roles of NO in the brain have been linked to activation of NMDA receptors. It has been known that NO is involved in synaptic plasticity, learning and memory formation, and the expression of behavioral sensitization and tolerance to psychostimulants (Noda *et al.*, 1996)

Opioid receptors belong to the G protein-coupled receptor superfamily. It is well known that opioid receptors transduce signals through pertussis toxin-sensitive G proteins to inhibit adenylyl cyclase, increase membrane K^+ conductance and reduce Ca^{2+} current (Childers, 1991; Rhim and Miller, 1994). Mitogen-activated protein kinase (MAPKs), which include extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK), are serine/threonine kinase that play critical role in cell growth and survival (Davis, 1993). Growth factors, cytokines and mitogens activate the ERK cascades, which in turn induce subsequent phosphorylation and activation of targets such as protein kinases, transcription factors and membrane proteins (Fukunaga and Miyamoto, 1998). Various studies provide arguments to support a critical role of protein phosphorylation and/or gene expression in opioid addiction (Nestler 1996; Koob *et al.*, 1998). A growing body of evidence suggests that the MAPKs are important for learning and memory and synaptic plasticity (Impey *et al.*, 1999). It is of interest to note that MAPK signals, especially ERK and p38, have been directly regulated by opioid receptors (Belcheva *et al.*, 1998; Zhang *et al.*, 1999). It has been reported that chronic administration of morphine induces increases of ERK activity in the ventral tegmental area, and the ERK activation in this region is associated with the morphine-induced increase in activities of tyrosine hydroxylase and rewarding (Berhow *et al.*, 1996; Ozaki *et al.*, 2004).

This study was undertaken to determine the antagonism of nalbuphine analgesia by AP1700, the inhibitory effects of AP1700 on the development of nalbuphine tolerance and physical dependence in rats, and the hepatic glutathione contents which are closely related to the degree of detoxification of morphinone, a novel metabolite of morphine (Nagamatsu *et al.*,1983). Also, we determined the inhibitory effect of AP1700 on the modulation of MAPK, NMDA receptor (NR1 subunit), nNOS expression in the rat brain regions.

MATERIALS AND METHODS

Materials

Rats (Sprague-Dawley, male) weighing 250-280g in a group of 10, were used in all experiments. The AP1700 is composed of 5 natural products (Sulfur precipitatum, Aconiti Tuber, Atractyloids Rhizoma Alba, cinnamon, Lonicerae Flos) was dissolved in distilled water and administered orally. Nalbuphine hydrochloride (Jeil Pharm., Seoul), and naloxone hydrochloride (Sigma) were dissolved in saline and administered to rat intraperitoneally.

Effects of AP1700 on nalbuphine analgesia and inhibition of analgesic tolerance development

In the test of nalbuphine antagonism by AP1700, the analgesic action of nalbuphine 10 mg/kg (i.p.) was estimated at 0, 30, 60 and 90 min by tail flick (D'Amour *et al.*, 1942) methods 1h after the oral administration of AP1700. To test nalbuphine tolerance, nalbuphine 10mg/kg was administered to rats once a day for a period of 6 days and AP1700 (250 mg/kg) was administered orally 1h prior to the injection of nalbuphine daily.

The tail flick latencies to thermal stimulation were determined in seconds prior to and at 0, 30, 60, and 90min after the nalbuphine injection. A value of 20sec was used as a cut-off point to avoid damage to the tail. The analgesic response for each rat was calculated by the following formula:

Percent Analgesia(%) =(Tt-To)/(Tc-To) ×100

Where To is the base line or pre-nalbuphine reaction time, Tt is the reaction time at t min after nalbuphine injection, and Tc is cut-off time. The base lines of tail flick latencies in different groups were around 3 ± 0.5sec. The analgesic effect was calculated and expressed as a percentage of the effect obtained in the control animals treated only with nalbuphine 10 mg/kg.

Measurement of the inhibition of naloxone-induced withdrawal

Additional groups of rat that had received the same nalbuphine and AP1700 as described in the development of nalbuphine tolerance were used in this experiment. The inhibition of

naloxone-induced withdrawal syndrome in nalbuphine-dependent rat was estimated by the observation of the withdrawal syndrome by naloxone 10 mg/kg (i.p.) on the seventh day, 7h after the final injection of nalbuphine. The withdrawal syndrome was induced by naloxone, and observed after placing animals on a plastic cage for 30min. The prototype of withdrawal syndrome was as below: wet-dog shake, rearing, escape behavior, penis licking, grooming, ptosis, diarrhea, teeth chattering.

Measurement of the level of pCREB, nNOS, NR1, pERK in nalbuphine withdrawal rat brain

Laemmli loading buffer was added to extracts (60 mg protein) and the samples were boiled for 4 min. Extracts were run on 12% SDS-PAGE gels and transferred electrophoretically to nitrocellulose. Blots were blocked in 5% skim milk and 2% bovine serum albumin in TBST for 3h and the membrane was probed with primary antibody at a dilution of 1:1000 (mouse anti-CREB, Transduction Lab.), 1:500 (rabbit anti-pCREB, Upstate), 1:2000 (mouse anti-nNOS, Transduction Lab.), 1:1000 (mouse anti-NR1, Pharmingen), 1:2000(mouse anti-pERK, Santa Cruz) for 24 h at 4°C. Blots were rinsed three times for 20 min in TBST, and incubated in horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz) or horse anti-rabbit IgG (Santa Cruz) at a 1:1000 dilution. The membrane was then rinsed 3 times for 5 min in TBST. Immunoreactivity was visualized using ECL chemiluminescence (Amersham Pharmacia Biotech).

Measurement of the hepatic glutathione contents in rat

Other additional groups of rat that had received the same nalbuphine and AP1700 as described in the development of nalbuphine tolerance were killed by decapitation on the seventh day. The liver was removed immediately. The glutathione concentration in the liver was determined by the method of Ellman as follows (Ellman, 1959); the wet liver was homogenized in 4 volumes of 0.5M sodium phosphate buffer, pH 7.4. For an estimation of reduced glutathione, the homogenized liver, 0.5ml was deproteinized by addition of 0.5 ml of 4% trichloroacetic acid containing 1mM Na-EDTA and centrifuged at 3000 x g for 5 min at 4°C. The supernatant(0.5 ml) was added to 4.5 ml of 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) and allowed to stand for 20min at room temperature. The reaction mixture was measured at absorbance 412 nm against blank.

Statistics

The data were expressed as mean ± S.E. The differences in the means for different responses in different treatment groups were analyzed by the Student's t-test.

RESULTS

Inhibition of analgesic tolerance development

No significant differences and antagonism of nalbuphine analgesia were observed in rat determined at 1h after administration of AP1700 by methods of tail flick (Fig. 1). In the test of the inhibitory effect of analgesic tolerance development, the analgesia of each group showed same tendency of tolerance.

Inhibition of naloxone-induced withdrawal

The inhibitory action of NA1700 on naloxone-induced withdrawal syndrome was significant in the wet-dog shake, rearing, and penis licking, but does not inhibit the escape behaviour, grooming, ptosis, and teeth chattering (Table I).

Immunoblot

The western blot was performed to examine the effect of AP1700 on the modulation of pCREB in the several brain regions. There was no significant change in CREB immunoblot on the cortex, hippocampus and striatum in nalbuphine tolerant or withdrawn rats (data not shown). However, the level of pCREB was significantly elevated in hippocampus by the treatment with nalbuphine or AP1700 but the level of pCREB was not significantly inhibited by the co-treatment with nalbuphine and AP1700 in the hippocampus (Fig. 2). Repeated treatment with nalbuphine or AP1700 did not modulate the expression of nNOS and NR1 in the tested brain regions (Fig. 3, Fig. 4).

Table I. Inhibition of withdrawal signs elicited in nalbuphine dependent rats by administration of AP1700.

	Saline	Ap1700	Nalbuphine	Nalbuphine + Ap1700
Escape behavior	0/10	0/10	1/10	1/10
Wet-Dog shake	2/10	2/10	10/10	3/10*
Rearing	1/10	2/10	9/10	3/10*
Penis licking	0/10	0/10	5/10	0/10*
Grooming	3/10	3/10	10/10	3/10*
Ptosis	0/10	0/10	6/10	1/10*
Diarrhea	0/10	0/10	4/10	0/10*
Teeth chattering	1/10	1/10	3/10	1/10

Rats were received nalbuphine (10 mg/kg, i.p.) and/or AP1700 (250 mg/kg, p.o.) for 6 days, and were challenged with naloxone (10 mg/kg, i.p.) 24h after the final injection of nalbuphine. Numbers denote the number of rats showing positive signs over the total number of rats tested for 30 min after injection of naloxone. *$P<0.05$, compared with the saline group by Fisher-exact test.

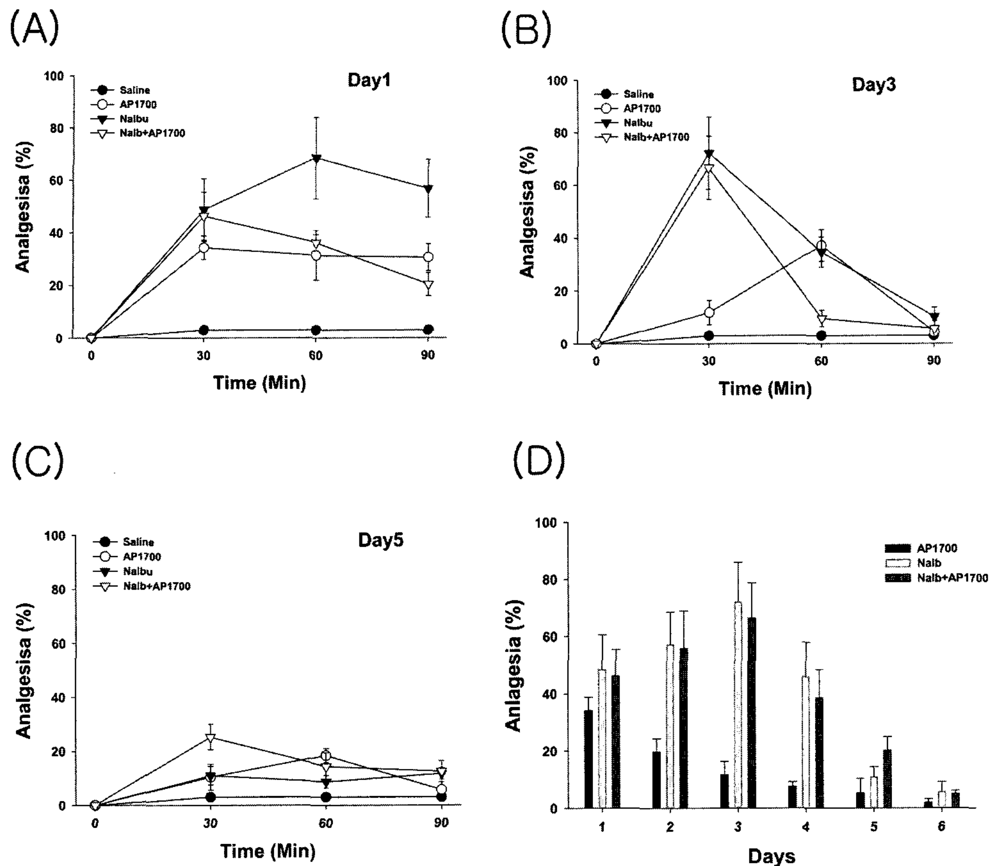

Fig. 1. Effect of AP1700 administered orally on the analgesic action of nalbuphine. Nalbuphine 10 mg/kg (i.p.) was administered to rat for 6days. AP1700 250 mg/kg were administered orally to rat 1hr prior to the injection of nalbuphine. The test of nalbuphine analgesia was estimated at 0, 30, 60, 90 minutes for 6days by tail flick methods (n=10). The inhibitory effects of AP1700 on the development of nalbuphine-induced tolerance were shown on day 1 (A), day 3 (B), and day 5 (C). Also, analgesic effect at 30 min for 6days was shown as histogram (D).

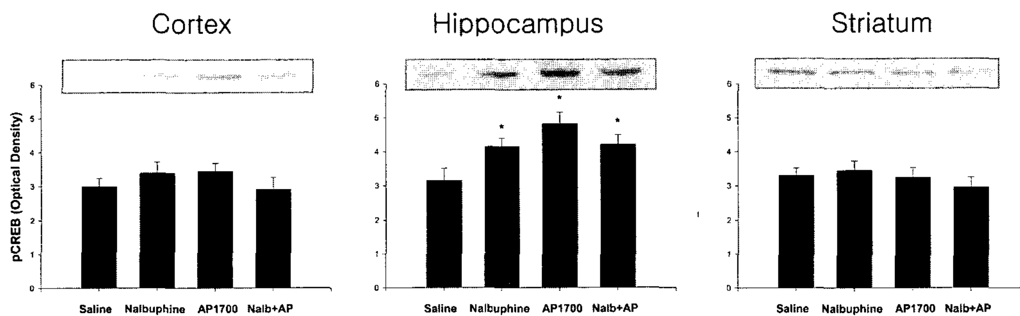

Fig. 2. Effect of AP1700 on the nalbuphine analgesia assessed the expression of pCREB in cortex, hippocampus, striatum. The pCREB protein was analyzed by Western blot, band intensities were quantified by densitometric imaging (n=4). Nalbuphine 10mg/kg i.p. was administered to rat for 6days. AP1700 250mg/kg were administered orally to rat 1hr prior to the injection of nalbuphine. Values are mean ± standard error of three experiments performed in triplicate. *p<0.05 from saline group.

Interestingly, co-treatment with nalbuphine and AP1700 decreased the expression of pERK in the cortex and striatum but not in the hippocampus (Fig. 5).

Measurement of the hepatic glutathione contents

The hepatic glutathione concentration (μ mol/g tissue) in the groups treated with AP1700 was slightly increased from 19.70 ± 1.42 in saline group to 22.58 ± 1.96 in 250mg/kg treated group. However, the glutathione level in the nalbuphine group was increased to 22.21 ± 3.00 μ mol/g tissue. The glutathione

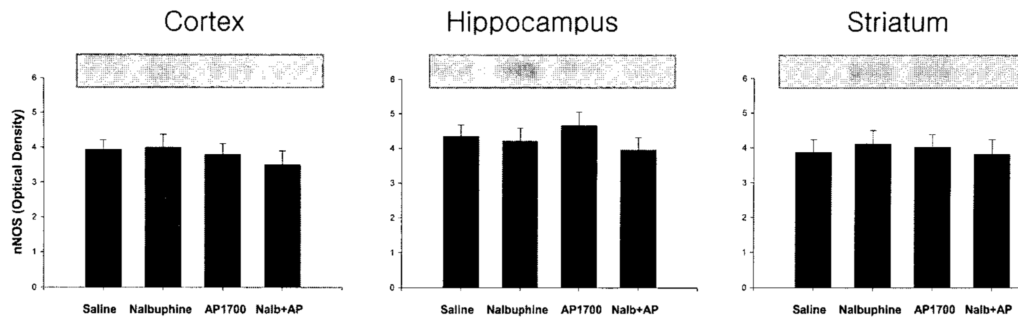

Fig. 3. Effect of AP1700 on the nalbuphine analgesia assessed the expression of nNOS in cortex, hippocampus, striatum (n=4). Nalbuphine 10 mg/kg i.p. was administered to rat for 6days. AP1700 250 mg/kg were administered orally to rat 1hr prior to the injection of nalbuphine. Values are mean ± standard error of three experiments performed in triplicate.

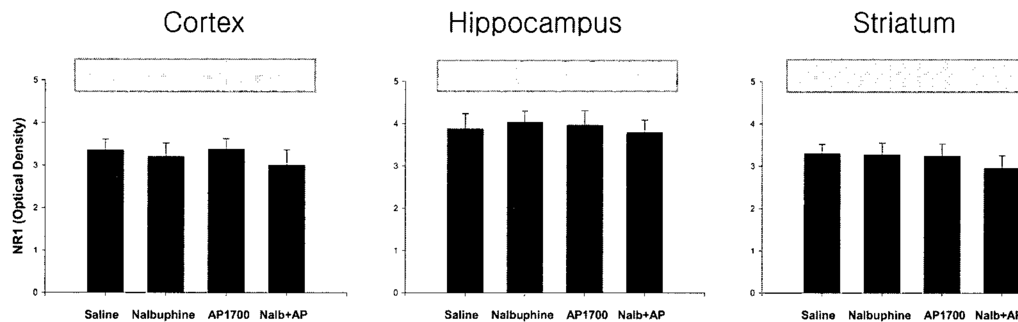

Fig. 4. Effect of AP1700 on the nalbuphine analgesia assessed the expression NR1 in cortex, hippocampus and striatum (n=4). Nalbuphine 10 mg/kg i.p. was administered to rat for 6days. AP1700 250 mg/kg were administered orally to rat 1hr prior to the injection of nalbuphine. Values are mean ± standard error of three experiments performed in triplicate.

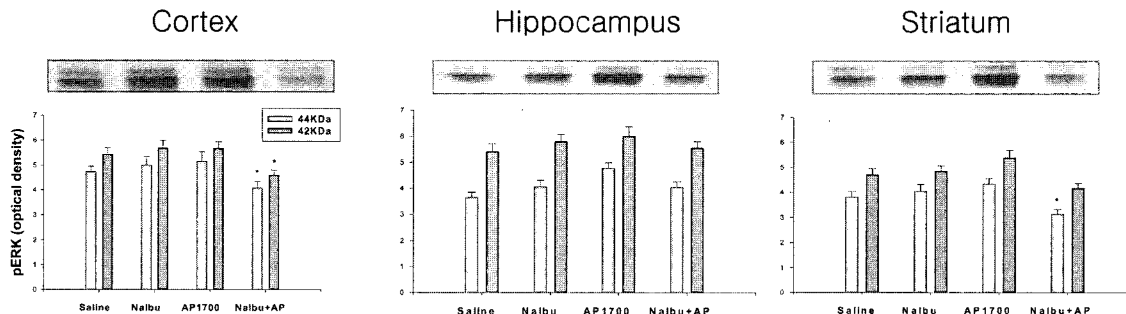

Fig. 5. Effect of AP1700 on the nalbuphine analgesia assessed the expression of pERK in cortex and hippocampus (n=4). Nalbuphine 10 mg/kg i.p. was administered to rat for 6days. AP1700 500 mg/kg were administered orally to rat 1hr prior to the injection of nalbuphine. Values are mean ± standard error of three experiments performed in triplicate. *p<0.05 from saline group respectively.

levels of the groups treated with nalbuphine and AP1700 were observed as 19.41 ± 2.70. So, there was no significant inhibitory effect in the hepatic glutathione level by the co-treatment with AP1700 (Fig. 6).

DISCUSSION

In this experiment, AP1700 inhibited the development of nalbuphine-induced physical dependence but did not show significant antagonism on the nalbuphine-induced tolerance in rats. The cellular mechanisms underlying inhibitory effect of AP1700 on the nalbuphine-induced withdrawal syndrome remain unknown. Western blot analysis of protein levels suggests that signal transduction system was not solely attributable to the nalbuphine-induced withdrawal syndrome. We hypothesize that the inhibitory effects of AP1700 on nalbuphine-induced physical dependence are closely related to the modulation of CREB (cyclic AMP response element-binding) protein

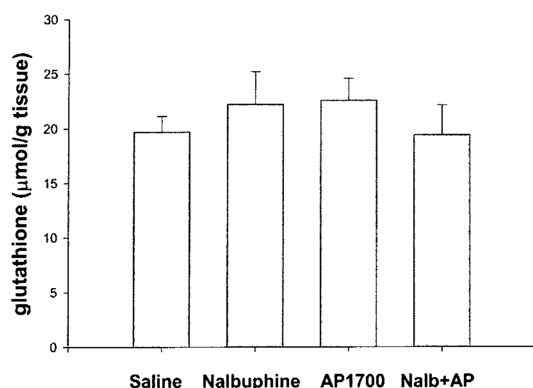

Fig. 6. Effect of AP1700 on the hepatic glutathione level in nalbuphine treated rats. Nalbuphine 10 mg/kg i.p. was injected into the rat for 6days. AP1700 250 mg/kg were administered orally to the respective group once a day 1hr prior to the nalbuphine injection for 6 days.

expression.

The CREB protein can activate transcription only when it is phosphorylated on a particular serine residue because phosphorylation of this residue permits CREB to interact with an adapter protein known as CREB-binding protein. CREB can be activated with the elevation of cAMP or Ca^{2+} by causing its phosphorylation at ser133. The cAMP activates PKA, whereas Ca^{2+} activates Ca^{2+}/calmodulin-dependent protein kinase, both of which phosphorylate ser133. The activation of a single transcription factor by convergent signaling pathways is particularly important in the nervous system because it may represent a mechanism for long-term neural adaptations, such as those underlying long-term memory, drug addiction, and fear conditioning (Nestler *et al.*, 2001). Increasing evidence indicates that chronic opiate-induced upregulation of the cAMP related signal system (adenylyl cyclase, CREB etc.) contributes to opiate tolerance, dependence, and withdrawal exhibited in locus coeruleus neurons (Nestler, 1992). This upregulated cAMP pathway can be reviewed as a homeostatic response of the neurons to persistent inhibition of the cells by opiates. It has been known that the upregulated cAMP system by abrupt removal of the opiate accounts for part of the withdrawal activation of the cells. However, the level of Gas mRNA was downregulated and the level of [^{3}H]forskolin was not significantly changed in butorphanol-withdrawal rats although the level of pCREB was elevated (Kim *et al.*, 2003). One of the possible explanations of this discrepancy is that the upregulation of pCREB is highly relied on the activation of NMDA receptor resulting in the elevation of intracellular Ca^{2+} and CaMK in opioid withdrawal.

In our experimental results, the level of pCREB was not significantly modulated by the co-treatment with nalbuphine and AP1700 although the level was decreased than that of AP1700 alone. Furthermore the level of NR1 and nNOS was not elevated by the treatment with nalbuphine. These two target subunit and enzyme have been thought as a key marker of morphine dependence (Oh *et al.*, 2000; Cuellar *et al.*, 2000). These discrepancies may denote the differential pharmacological action of mu-opioid receptor favoring agonist (morphine) and kappa-opioid receptor favoring agonist (nalbuphine). Interestingly, the expression of pERK was downregulated in the treatment with nalbuphine and AP1700. It has been reported that chronic administration of morphine induces increases of ERK activity in the ventral tegmental area (VTA), and the ERK activation in this region is associated with the morphine-induced rewarding (Berhow *et al.*, 1996; Ozaki *et al.*, 2004). It has been suggested that a sustained reduction in the ERK-dependent signaling pathway in dopamine cells of the VTA may be implicated in the suppression of the morphine-induced rewarding effect under neuropathic pain (Ozaki *et al.*, 2004). However, it needs some more experiment to explain the correlation with antagonism of dependence and pERK suppression. It could be presumed that AP1700 ameliorated the drug abuse to inhibit the psychological rewarding via downregulation of pERK expression as well as inhibit the physical dependence. In fact, AP1700 suppressed the methamphetamine-induced conditioned place preference (rewarding) by oral administration with a similar dose in mice (data not shown). However, the level of pCREB and pERK was elevated by the treatment of AP1700 alone in the hippocampus. This up-regulation could be helpful to enhance the memory performance in the hippocampus, although it is not easy to explain why this level was increased.

In the liver of mice, a portion of morphine was metabolized into morphinone which was a novel metabolite of morphine, and had 9 times the toxicity of morphine but half the analgesic activity of morphine, based on LD_{50} and ED_{50} values in each mouse (Nagamatsu *et al.*, 1982). It was also reported that morphinone could function as a key substance of morphine tolerance since morphinone binds covalently sulfhydryl groups of opiate receptors, and inactivates irreversibly opiate binding sites, thus blocking the analgesic effect of morphine (Nagamatsu *et al.*, 1982). Accordingly, these facts suggest that control of the morphinone production is a very important problem on the development of tolerance. An aliquot of morphinone conjugated with glutathione was closely related to the detoxification process. The other aliquot of morphinone was metabolized into morphinone-protein SH conjugate concerned with the develop-

ment of morphine-induced tolerance and physical dependence by covalent binding to the sulfhydryl group of opiate receptor (Nagamatsu *et al.*, 1983). However, we observed that nalbuphine did not induce significant elevation of glutathione in the repeated treatment with nalbuphine. These results suggest that nalbuphine shows the different pharmacokinetic as well as pharmacodynamic actions from those of morphine. In fact, sulfur precipitatum, one of the components of AP1700 did not inhibit the nalbuphine-induced withdrawal syndrome when it was pretreated with nalbuphine (data not shown). Collectively, AP1700 shows the inhibitory action to the withdrawal syndrome of nalbuphine and modulates the pERK level in the brain regions. This is the first observation to date in inhibition of the nalbuphine-induced withdrawal syndrome by using agent or formula. This result suggests that AP1700 may be developed as a therapeutic formula in treatment of opiate abuse.

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