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Inhibitory Action of the Ginseng Total Saponin on the Nalbuphine-Induced Tolerance and Withdrawal Syndrome

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Abstract : This study was undertaken to determine the antagonism of the ginseng total saponin (GTS) on the development of nalbuphine-induced tolerance and physical dependence. GTS is known to have antinarcotic action with a dose of 100mg/kg (i.p.) in rats. GTS significantly inhibits the development of nalbuphine-induced physical dependence as well as the tolerance. The level of pCREB was elevated in the striatum by the chronic treatment with nalbuphine or GTS, however, the elevation of pCREB was inhibited by the GTS co-treatment. It has been suggested that NMDA receptor and/or NO is involved in the penomena of opioid dependence and withdrawal. However, the level of nNOS and NR1 was not modulated by the treatment with nalbuphine or GTS on the cortex, hippocampus and striatum in the rat brain. These results suggest that the GTS could be used to ameliorate the nalbuphine tolerance and withdrawal symptoms.

Key words : nalbuphine, analgesia, tolerance, dependence, pCREB

INTRODUCTION

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.[1,2] 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.[3] The nalbuphine has a low dependence profile, possibly related to its ability to an antagonize morphine and other mu opioid drugs.[3] For the prevention of opioid-related side effects, both nalbuphine and naloxone can effectively decrease the incidence of respiratory depression, nausea, vomiting, and pruritus.[4]

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 morphine 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

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suggested that NMDA receptor and/or NO is involved in the phenomena of opioid dependence and withdrawal.[5,6] When non-competitive NMDA receptor antagonists such as ketamine, MK-801, and dextromethorphan are administered immediately prior to naloxone-precipitated morphine withdrawal, signs of withdrawal syndrome are attenuated.[7,8] 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.[9]

It has been known that the inhibitory effects of ginseng on the development of morphine tolerance and dependence, development of reverse tolerance of methamphetamine, and development of cocaine-induced reverse tolerance and dopamine receptor supersensitivity.[10,11,12] This study was undertaken to determine the antagonism of nalbuphine analgesia by GTS, the inhibitory effects of GTS 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.[13] Also, we determined the inhibitory effect of GTS on the nNOS expression, modulation of NMDA receptor (NR1 subunit), and pCREB in the rat brain regions.

MATERIALS AND METHODS

Materials

Male Sprague-Dawley rats (Folas, Eumsung) weighing 250-280 g in a group of 10, were acclimatized for 1 week with free access to rat chow and tap water. The temperature (24±3°C) and light (12 h dark) of the housing environment were maintained constantly. The ginseng total saponin (GTS) was obtained from KT&G Central Research Institute 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 GTS on nalbuphine analgesia and inhibition of analgesic tolerance development

In the test of nalbuphine antagonism by GTS, the analgesic action of nalbuphine 10 mg/kg (i.p.) was estimated at 0, 30, 60 and 90 min by tail flick methods[14] 1h after the administration of GTS (i.p.). To test nalbuphine tolerance, nalbuphine 10 mg/kg was administered to rats once a day for a period of 6 days and GTS (100 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 90 min after the nalbuphine injection. A value of 20 sec 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:

$$\text{Percent Analgesia}(\%) = (Tt\text{-}To) / (Tc\text{-}To) \times 100$$

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±0.5 sec. 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 GTS 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, 7 h after the final injection of nalbuphine. The withdrawal syndrome was induced by naloxone, and observed after placing animals on a plastic cage for 30 min. 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 in nalbuphine withdrawal rat brain

Laemmli loading buffer was added to extracts (60 g 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 3 h 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) 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 GTS 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[15]; 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 × g for 5 min at 4°C. The supernatant (0.5ml) was added to 4.5 ml of 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) and allowed to stand for 20 min 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 using ANOVA followed by the Newman-Keuls's post hoc test.

RESULTS

1. Inhibition of analgesic tolerance development

GTS shows the inhibitory action to the nalbuphine-induced analgesic tolerance. Analgesia was observed in

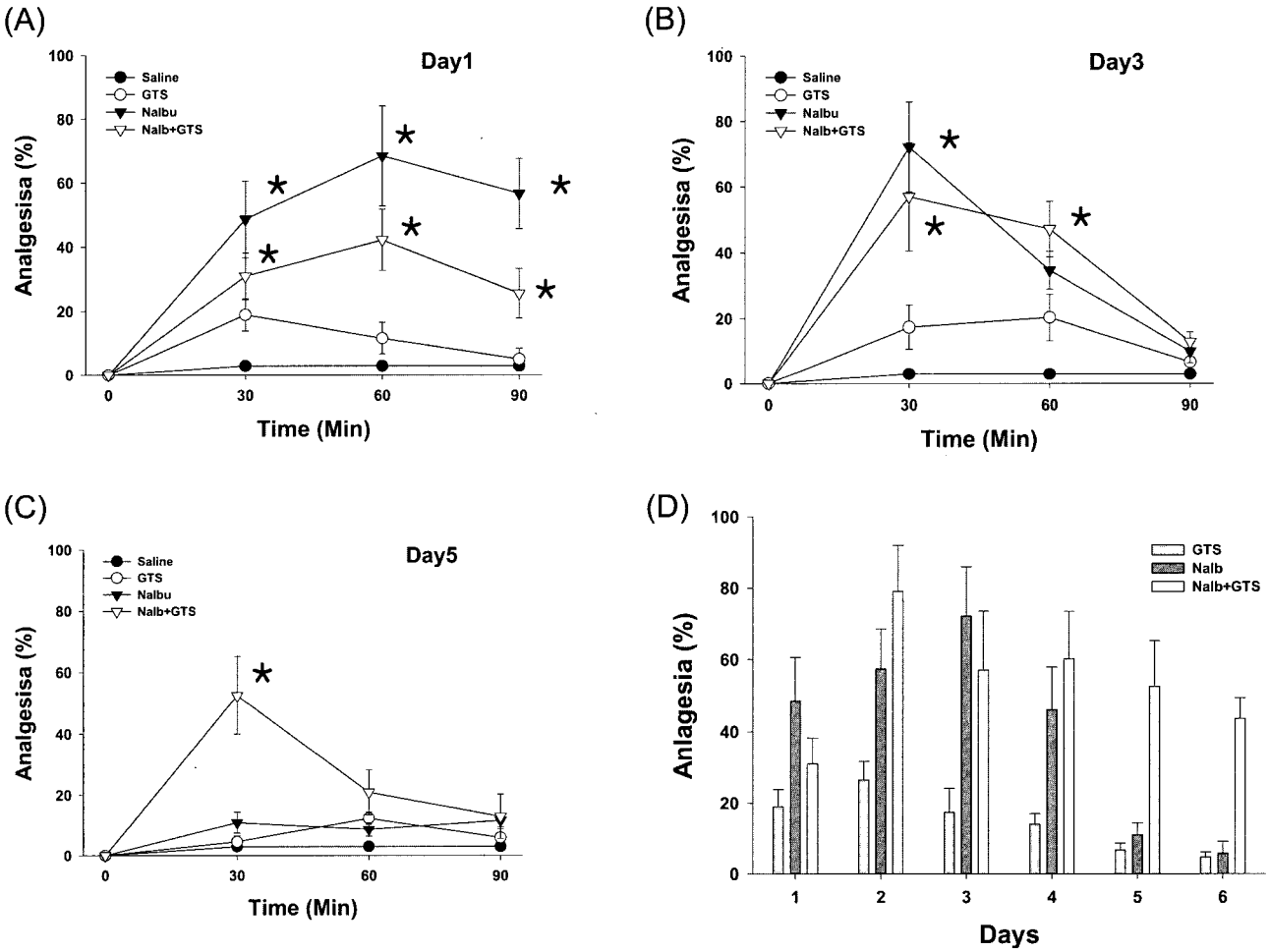

Fig. 1. Effect of GTS on the analgesic action of nalbuphine. Nalbuphine 10mg/kg (i.p.) was administered to rat for 6days. GTS 100 mg/kg was administered (i.p.) to rat 1hr prior to the injection of nalbuphine. The test of nalbuphine analgesia was estimated at 0, 30, 60, 90 minutes for 6days by tail flick methods (n=10). The inhibitory effects of GTS 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). *$p<0.05$ from respective saline group.

rat determined at 1 h after administration of GTS by methods of tail flick (Fig. 1). In the test of the inhibitory effect of analgesic tolerance development, the analgesia of each group showed relatively high in the GTS co-administration. Especially, analgesic effect of nalbuphine was almost abolished by the repeated treatment at day 5. However, the analgesic effect of nalbuphine was maintained by the co-administration of GTS with nalbuphine.

2. 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 1).

3. Immunoblot

The western blot was performed to examine the effect of GTS 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).

Table 1. Inhibition of withdrawal signs elicited in nalbuphine dependent rats by administration of GTS.

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

Rats were received nalbuphine (10 mg/kg, i.p.) and/or GTS (100 mg/kg, i.p.) for 6 days, and were challenged with naloxone (10 mg/kg, i.p.) 24 h 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.

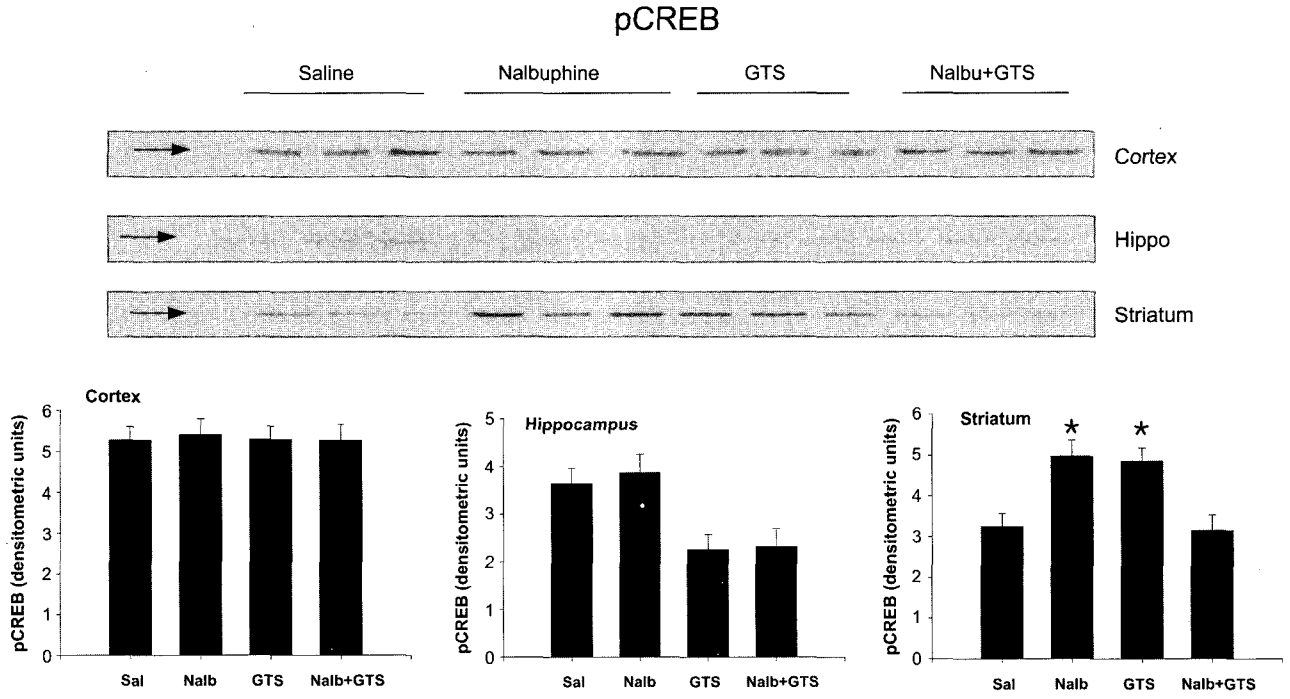

Fig. 2. Effect of GTS on the nalbuphine analgesia assessed the expression of pCREB in cortex, hippocampus, striatum. The pCREB protein was analyzed by Western blot, band intensities were quantified by densitometric imaging (n=4). Nalbuphine 10 mg/kg i.p. was administered to rat for 6days. GTS 100 mg/kg was administered (i.p.) 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.

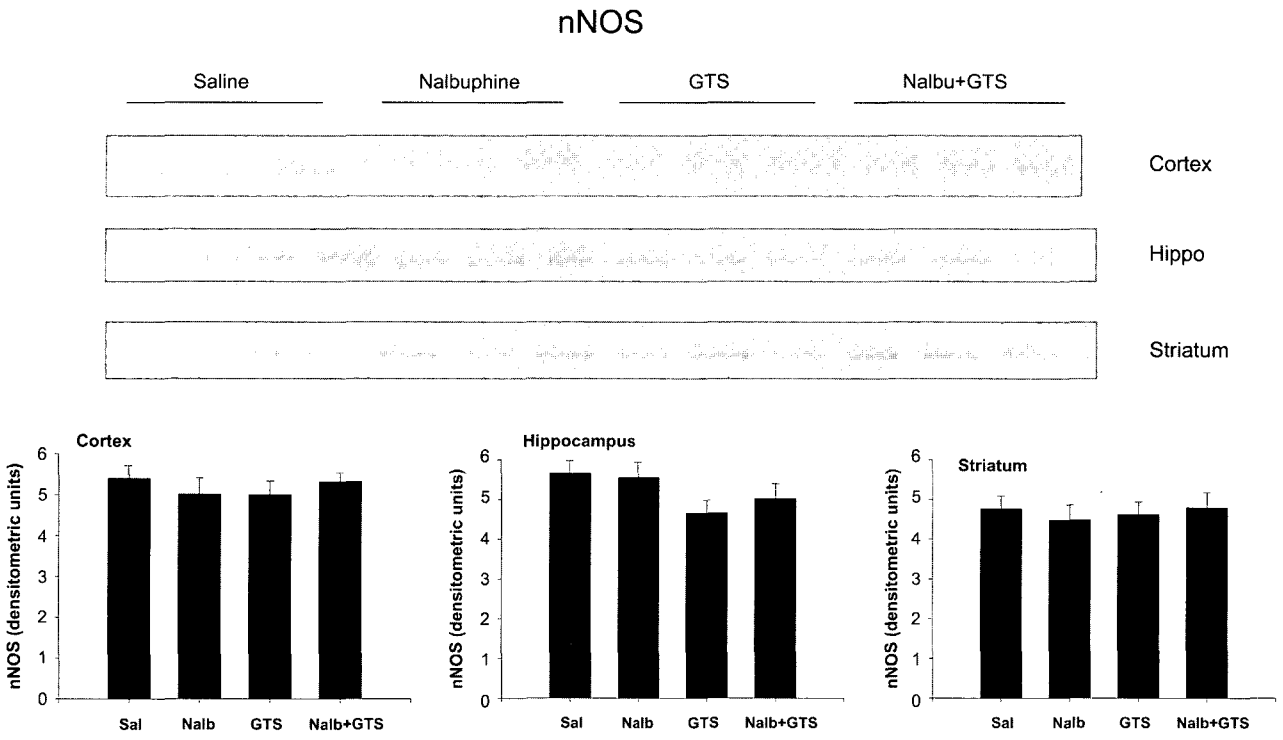

Fig. 3. Effect of GTS on the nalbuphine analgesia assessed the expression of nNOS in cortex, hippocampus, striatum (n=4). Nalbuphine 10 mg/kg i.p. was administered to rat for 6days. GTS 100 mg/kg was administered (i.p.) to rat 1hr prior to the injection of nalbuphine. Values are mean ±standard error of three experiments performed in triplicate.

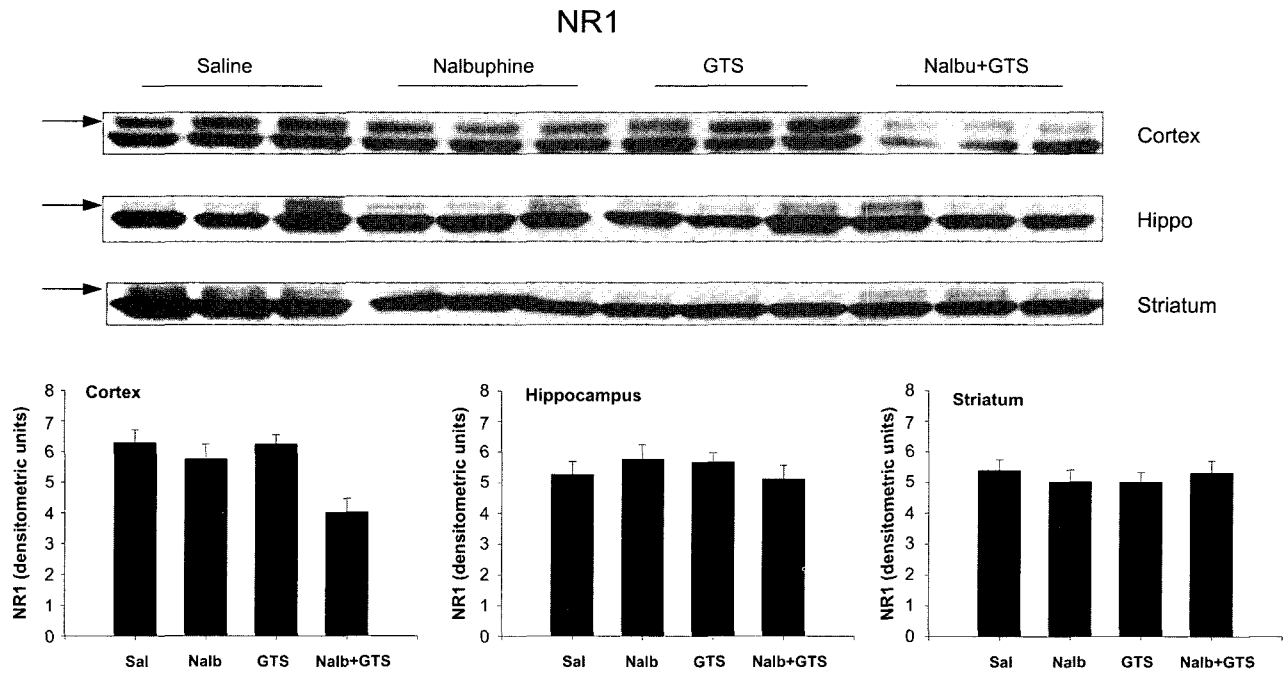

Fig. 4. Effect of GTS on the nalbuphine analgesia assessed the expression NR1 in cortex, hippocampus and striatum (n=4). Nalbuphine 10 mg/kg i.p. was administered to rat for 6days. GTS 100 mg/kg was administered (i.p.) to rat 1hr prior to the injection of nalbuphine. Values are mean ±standard error of three experiments performed in triplicate.

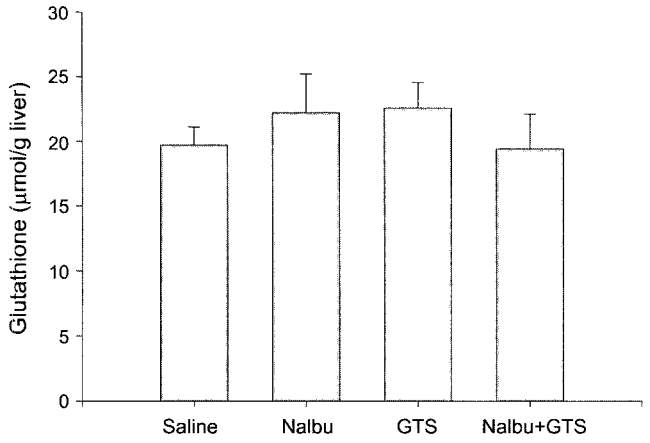

Fig. 5. Effect of GTS on the hepatic glutathione level in nalbuphine treated rats. Nalbuphine 10 mg/kg i.p. was injected into the rat for 6days. GTS 100 mg/kg was administered (i.p.) to the respective group once a day 1hr prior to the nalbuphine injection for 6days.

However, the level of pCREB was significantly elevated in striatum by the treatment with nalbuphine or GTS but the level of pCREB was significantly inhibited by the co-treatment with nalbuphine and GTS in the striatum (Fig. 2). Repeated treatment with nalbuphine or GTS did not modulate the expression of nNOS and NR1 in the tested brain regions (Fig. 3, Fig. 4).

4. Measurement of the hepatic glutathione contents

The hepatic glutathione concentration (mol/g tissue) in the groups treated with GTS was slightly increased from 19.70 ± 1.42 in saline group to 22.40 ± 1.90 in 100 mg/kg treated group. However, the glutathione level in the nalbuphine group was increased to 22.25 ± 2.95 mol/g tissue. The glutathione levels of the groups treated with nalbuphine and GTS were observed as 19.40 ± 3.00. So, there was no significant inhibitory effect in the hepatic glutathione level by the co-treatment with GTS (Fig. 5).

DISCUSSION

In this experiment, ginseng total saponin (GTS) inhibited the development of nalbuphine-induced physical dependence as well as the tolerance in rats. The cellular mechanisms underlying inhibitory effect of GTS 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 GTS on nalbuphine-induced physical dependence and tolerance are closely related to the modulation of CREB (cyclic AMP response element-binding) protein 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.[16] 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.[17] 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 Gs 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.[18] 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 significantly modulated by the co-treatment with nalbuphine and GTS in the striatum although the level was increased by the treatment with GTS alone. 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.

However, 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.[5,6] These discrepancies may denote the differential pharmacological action of mu-opioid receptor favoring agonist (morphine) and kappa-opioid receptor favoring agonist (nalbuphine).

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.[19] 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.[20] 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 development of morphine-induced tolerance and physical dependence by covalent binding to the sulfhydryl group of opiate receptor.[13] 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. Collectively, GTS shows the inhibitory action to the withdrawal syndrome of nalbuphine and modulates the pCREB level in the brain regions. This is the first observation to date in inhibition of the nalbuphine-induced withdrawal syndrome as well as tolerance by using agent or formula. This result suggests that ginseng may be developed as a therapeutic formula in treatment of opiate abuse.

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