Research article

Morphine dependence is attenuated by red ginseng extract and ginsenosides Rh2, Rg3, and compound K

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A B S T R A C T

Background: Red ginseng and ginsenosides have shown plethoric effects against various ailments. However, little is known regarding the effect of red ginseng on morphine-induced dependence and tolerance. We therefore investigated the effect of red ginseng extract (RGE) and biotransformed ginsenosides Rh2, Rg3, and compound K on morphine-induced dependence in mice and rats.
Methods: While mice were pretreated with RGE and then morphine was injected intraperitoneally, rats were infused with ginsenosides and morphine intracranially for 7 days. Naloxone-induced morphine withdrawal syndrome was estimated and conditioned place preference test was performed for physical and psychological dependence, respectively. Western blotting was used to measure protein expressions.
Results: Whereas RGE inhibited the number of naloxone-precipitated jumps and reduced conditioned place preference score, it restored the level of glutathione in mice. Likewise, ginsenosides Rh2, Rg3, and compound K attenuated morphine-dependent behavioral patterns such as teeth chattering, grooming, wet-dog shake, and escape behavior in rats. Moreover, activated N-methyl-D-aspartate acid receptor subunit 1 and extracellular signal-regulated kinase in the frontal cortex of rats, and cultured cortical neurons from mice were downregulated by ginsenosides Rh2, Rg3, and compound K despite their differential effects.
Conclusion: RGE and biotransformed ginsenosides could be considered as potential therapeutic agents against morphine-induced dependence.

1. Introduction

Benefits of opioid therapy for acute and chronic pain leads to their frequent use; however, opioids such as morphine have been associated with increased abuse, misuse, and overdose [1] accompanied by the risk of tolerance, dependence, and illicit diversion [2]. Pharmacological studies conducted so far have identified a number of signaling proteins involved in morphine-induced tolerance and dependence, including the N-methyl-D-aspartate acid receptor (NMDAR), nitric oxide synthase [3], protein kinase C, protein kinase A (PKA), calcium (Ca^{2+})/calmodulin-dependent kinase II, delta-opioid receptor, and regulators of G-protein signaling proteins. The G protein-coupled μ-opioid receptor and NMDAR-mediated signals work together in a sequential and interconnected manner to ultimately induce μ-opioid receptor desensitization [4–6]. Extracellular signal-regulated kinases (ERKs) have been shown to

be activated [7–9], and NMDAR subunit 1 (NR1) modulation was reported in morphine dependence [10].

A major goal of research on drug abuse is to develop effective treatments to deal with long-term behavioral disorders, especially reinstatement induced by abused drugs such as morphine. In this regard, phytochemicals obtained from medicinal herbs possess the capability to plague molecular mechanisms of tolerance, dependence, and addiction induced by abused drugs [11], or to enhance processes of their detoxification in the liver [12] and increase the physical and intellectual work capacity by their actoprotective effect [13]. Red ginseng and its active components, ginsenosides, have shown plethoric effects against various ailments [14]. It is well known that ginsenosides can be biotransformed by intestinal microflora and the metabolites would be absorbed into blood vessels. While incubation of ginseng protopanaxadiol saponins (Rb1, Rb2, and Rc) with human intestinal bacteria gives rise to

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compound K, ginsenoside Rg3 incubation confers Rh2 as a major metabolite [15].

Ginsenosides Rh2, Rg3, and compound K have been reported for apoptosis [16,17] and anticancer [18−20], anti-inflammatory [21,22], and antidepressant-like effects [23]. Previous reports indicated that ginsenosides with different structures have antagonizing properties in the regulation of morphine-induced reinforcement [24]. Despite the potential effects of ginsenosides on morphine dependence and tolerance reported so far, little is known regarding the effects of red ginseng extract (RGE) on morphine-induced behavioral disorders. Moreover, there is paucity of data pertaining to the effects of biotransformed ginsenosides such as Rh2, Rg3, and compound K on activated NR1 and ERK1/2. Therefore, this study revealed that RGE and ginsenosides (Rh2, Rg3, and compound K) ameliorate morphine-induced abnormal behaviors with restoration of hepatic glutathione level. Expressions of phosphorylated NR1 and ERK in the frontal cortical tissues of rats were also attenuated by Rh2, Rg3, and compound K.

2. Materials and methods

2.1. Materials

RGE was obtained from KT&G Central Research Institute in Korea, morphine hydrochloride from Myungmun Pharm (Seoul, Korea), and naloxone hydrochloride from Sigma-Aldrich (St. Louis, MO, USA); compound K, 20(S)-ginsenoside Rg3, and 20(S)-ginsenoside Rh2 were prepared as described previously [15]. All other chemicals, reagents, and antibodies were obtained from Sigma-Aldrich.

2.2. Animals and treatments

Both C57BL/6 male mice and male Sprague-Dawley rats were obtained from Daehan Biolink (Eumsung, Korea). Animals were housed and acclimatized (1 wk) on a 12-h light−dark cycle and maintained at $24 \pm 3°C$ with free access to chow and water. All animal procedures were in accordance with the Institutional Animal Care and Use Committee of Ewha Womans University, School of Medicine.

2.3. Treatments in mice

Mice (23 ± 2 g) were randomly assigned to each group and were given saline, morphine (10 mg/kg, i.p.), or both morphine and RGE (250 mg/kg and 500 mg/kg, p.o.) daily for 7 d. RGE was administered 30 min prior to the injection of morphine. Naloxone hydrochloride (5−10 mg/kg, i.p.) was injected 6 h after the final morphine injection for induction of morphine withdrawal syndrome in mice and rats.

2.4. Treatments in rats

Rats (220−240 g) were implanted with guide cannulae for drug infusion. Rats were anesthetized by intramuscular injection of ketamine (50 mg/kg) and xylazine (1 mg/kg), before performing standard stereotaxic surgery on a Kopf stereotaxic frame. A 21-gauge stainless-steel cannula was implanted in the right lateral ventricle (L: 1.3 mm; A−P: −0.5 mm; and D−V: −4.3 mm) of the rat brain with the bregma chosen as the stereotaxic reference point. The cannula was held in place with rapid-setting dental acrylic (Lang Dental Mfg. Co., Chicago, IL, USA) anchored to the skull by a plastic protective cap. Rats were allowed 1 wk for recovery before implantation of osmotic mini-pumps. The mini-pump was implanted subcutaneously as described previously, with minor modifications [15]. Briefly, under ether anesthesia, a small cut was made behind the ears of a rat, and the subcutaneous space was expanded with hemostatic forceps. Saline vehicle or ginsenoside was filtered through a 0.2 μm sterile syringe filter and was then used to fill an osmotic mini-pump (Alzet 2ML 1; Alza, Palo Alto, CA, USA). The mini-pump, which contains ginsenoside Rg3, Rh, or compound K, was implanted and connected directly to the morphine osmotic pump via 6-cm long PE-60 polyethylene tubing. The infusion rate was 10 μg/10 μL/h for 7 d. The incision on the back was closed with cyanoacrylate glue, and dental acrylic was layered on top of the polyethylene tube.

2.5. Measurement of morphine withdrawal syndrome

Morphine withdrawal syndrome in mice was induced by an injection of naloxone (5 mg/kg). Immediately after naloxone injection, mice were placed into individual observation cylinders (25 cm in diameter and 50 cm in height), and the frequency of jumps of each mouse was observed for 30 min. In rats, naloxone-induced morphine withdrawal syndrome was estimated 6 h after stopping the morphine infusion. The withdrawal syndrome was initiated by naloxone (10 mg/kg, i.p.) and rats were kept in plastic cages for 30 min to observe withdrawal symptoms such as wet-dog shake, escape behavior, grooming, and teeth chattering.

2.6. Conditioned place preference test

Conditioned place preference (CPP) test was used to evaluate the development of morphine-induced psychological dependence in mice. The CPP apparatus was composed of two square-based plexiglass compartments, one with white and the other with black walls, which could be separated by guillotine doors. A computer-based video-tracking system was used to record and analyze the behavioral data. The CPP test consisted of three phases: preconditioning, conditioning, and postconditioning. On the 1st day (Day 1) of the preconditioning phase, all mice were individually placed into the apparatus for 15 min. The guillotine doors were raised, allowing the animal a free access to both compartments. On the 2nd day (Day 2), the time spent in each chamber was recorded for 15 min and analyzed. The conditioning phase occurred over a period of 6 d. During this phase, the guillotine doors were closed. Each day, half of the mice in each group were confined to the black compartment and the other half to the white compartment. Mice were trained for 6 consecutive days. On Days 3, 5, and 7, mice received the drug just prior to being confined for 30 min in the compartment that they less preferred. On Days 4, 6, and 8, mice received saline just prior to being confined for 30 min to the compartment that they favored. During the postconditioning phase (Day 9), the guillotine doors were raised, and the untreated rats were placed in the tunnel in the central part of the apparatus. The time that rats spent in each compartment was recorded for 15 min. CPP scores were expressed as the difference in the preconditioning and postconditioning phases. All experiments were carried out between 2 and 5 PM.

2.7. Measurement of liver glutathione contents in mice

Mice were killed by decapitation on the 7th day of the experiment. The liver was removed immediately, and glutathione concentration was determined using Ellman method as follows: wet liver was homogenized in four volumes of 0.5M sodium phosphate buffer, pH 7.4, and then 0.5 mL was deproteinized by addition of 0.5 mL of 4% trichloroacetic acid containing 1mM Na-EDTA and centrifuged at 3,000g for 5 min at 4°C. The supernatant (0.5 mL) was added to 4.5 mL of 0.1mM 5,5′-dithiobis(2-nitrobenzoic acid) and allowed to stand for 20 min at room temperature. The reaction mixture was measured at 412 nm absorbance.

2.8. Primary cortical culture

Cortical cell culture was prepared from C57BL/6 mice embryo at a gestational age of 15 d. The brain was dissected; cortical tissues were taken out and dissociated in ice-cold buffer to prepare cell suspension, which was centrifuged at 1,000 rpm for 5 min, and then cell pellets were resuspended in Minimum Essential Medium (MEM) (Gibco BRL, Rockville, MD, USA) supplemented with 5% heat-inactivated fetal calf serum (Gibco BRL); the cells were cultured in an incubator at 5% CO_2 at 37°C for 7 d. Then, the cells were treated with 10 μM cytosine arabinofuranoside (Ara C) to reduce the growth of contaminating non-neuronal cells. The cortical neuronal cells were cultured in MEM containing 10% horse serum without glutamine.

2.9. Western immunoblotting

Protein concentrations in whole cell lysates or brain tissues were determined using a protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Sample buffer (4×) was added to the extract and boiled for 15 min at 100°C. Proteins (30−60 μg) underwent electrophoresis on 10% or 8% sodium dodecyl sulfate polyacrylamide gel and were transferred to polyvinylidene difluoride membrane. The blots were then blocked with 5% fat-free dry milk-tris-buffered saline containing 0.1% Tween-20 (TBST) buffer for 1 h and incubated with primary antibodies (ERK1/2, 1:1,000; pERK1/2, 1:2,000; NR1, 1:1,000; pNR1, 1:1,000; Santa Cruz Biotechnology, CA, USA) at 4°C overnight. After washing three times with TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000) in 3% fat-free dry milk-TBS for 1 h at room temperature. The blots were rinsed again three times with TBST, and the transferred proteins were incubated with the enhanced chemiluminescent detection kit according to the manufacturer's instructions and visualized with Imagequant LAS 4000 (Fujifilm Life Science, Tokyo, Japan).

2.10. Statistical analysis

All values were expressed as mean ± standard deviation values. Results were subjected to a one-way analysis of the variance by the Newman−Keuls multiple comparison test. Differences with $p < 0.05$ and $p < 0.01$ were considered statistically significant. All analyses were performed using SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. RGE reduced morphine-related physical dependence in mice

Morphine-induced physical dependence is associated with jumping behavior after injection of naloxone (5 mg/kg) in mice. Here, we observed that morphine-treated groups of mice showed the highest frequency of jumping (40) within 30 min compared with the groups treated with saline or RGE (500 mg/kg) only. RGE pretreatment at a dosage of 250 mg/kg reduced the number of jumps by half in morphine-treated mice, and this effect was highly diminished when the mice were subjected to 500 mg/kg RGE (Fig. 1).

3.2. RGE ameliorated morphine-induced psychological dependence in mice

One of the withdrawal symptoms of morphine injection is psychological dependence. To determine if mice pretreated with RGE and then injected with morphine were less likely to develop psychological dependence, the CPP test was used. Mice treated with morphine showed a higher CPP test score (3 times) than those
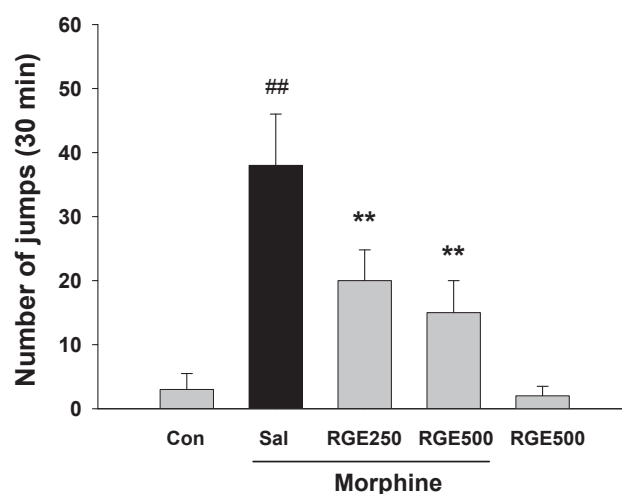

Fig. 1. Effects of red ginseng extract on naloxone-precipitated jumping behavior in morphine-dependent mice. RGE (250 mg/kg and 500 mg/kg, p.o.) were administered 30 min before morphine injection (10 mg/kg, i.p., $n = 10$) for 7 d. On the 7th day, naloxone (5 mg/kg, i.p.) was injected 6 h after final morphine administration. The number of jumps in 30 min was counted after naloxone injection. Data were expressed as mean ± SD ($n = 10$). ## $p < 0.001$ in comparison with the control group. ** $p < 0.01$ in comparison with the saline (morphine only) group. Con, control; RGE, red ginseng extract; Sal, saline; SD, standard deviation.

treated with RGE at 250 mg/kg. RGE pretreatment at a dose of 500 mg/kg in morphine-injected mice showed a similar effect to that of the untreated control (Fig. 2).

3.3. RGE restored the level of liver glutathione in morphine-treated mice

It was reported that hepatic glutathione, which is involved in the detoxification of chemical compounds, significantly decreased after intraperitoneal injection of morphine [25]. Therefore, we examined the level of this antioxidant substance in mice treated with RGE before exposure to morphine. Mice treated with
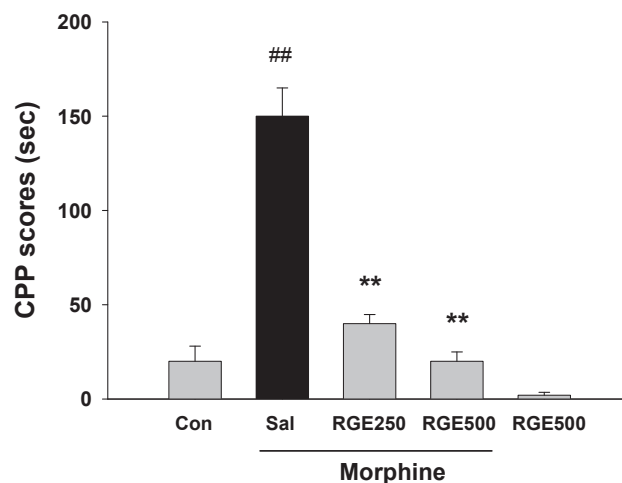

Fig. 2. The effect of RGE pretreatment against morphine-induced psychological dependence in mice. Mice were treated with RGE (250 mg/kg and 500 mg/kg, i.p.), morphine (10 mg/kg, i.p.), or RGE and morphine. The time that mice spent in each compartment of the CPP apparatus was recorded for 15 min. The CPP scores were calculated by taking the difference between preconditioning and postconditioning phases. Data were expressed as mean ± SD ($n = 8$). ## $p < 0.05$ compared with the control group (each group, $n = 8$). ** $p < 0.01$ compared with the morphine-only group. CPP, conditioned place preference; Con, control; RGE, red ginseng extract; Sal, saline; SD, standard deviation.

morphine alone at 10 mg/kg showed a marked fall in glutathione level; however, this was restored during pretreatment of RGE at 250 mg/kg and 500 mg/kg. Treatment of mice with RGE at a dose of 500 mg/kg alone showed neither an increase nor a decrease in the level of glutathione (Fig. 3).
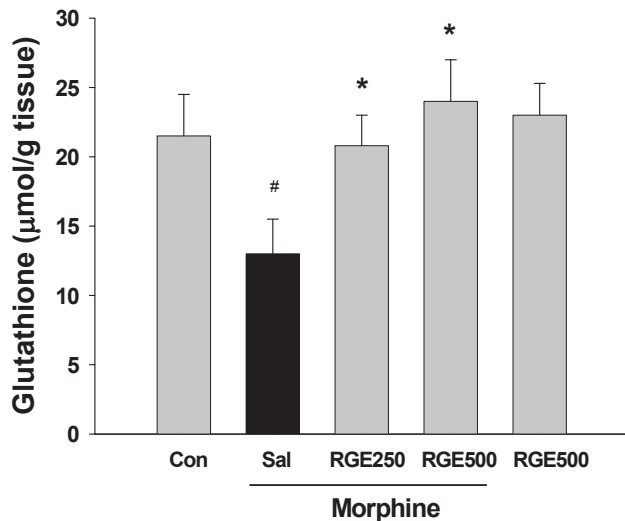

Fig. 3. Restorative effect of RGE on liver GSH content in morphine-treated mice. Mice were treated with morphine (10 mg/kg), morphine plus RGE (250 mg/kg and 500 mg/kg, i.p.), and RGE 500 mg/kg, i.p.). Data were expressed as mean ± SD ($n = 8$). $^{#} p < 0.05$ compared with the control group. $^{*} p < 0.05$ compared with the saline (morphine-only) group. Con, control; GSH, glutathione; RGE, red ginseng extract; Sal, saline; SD, standard deviation.
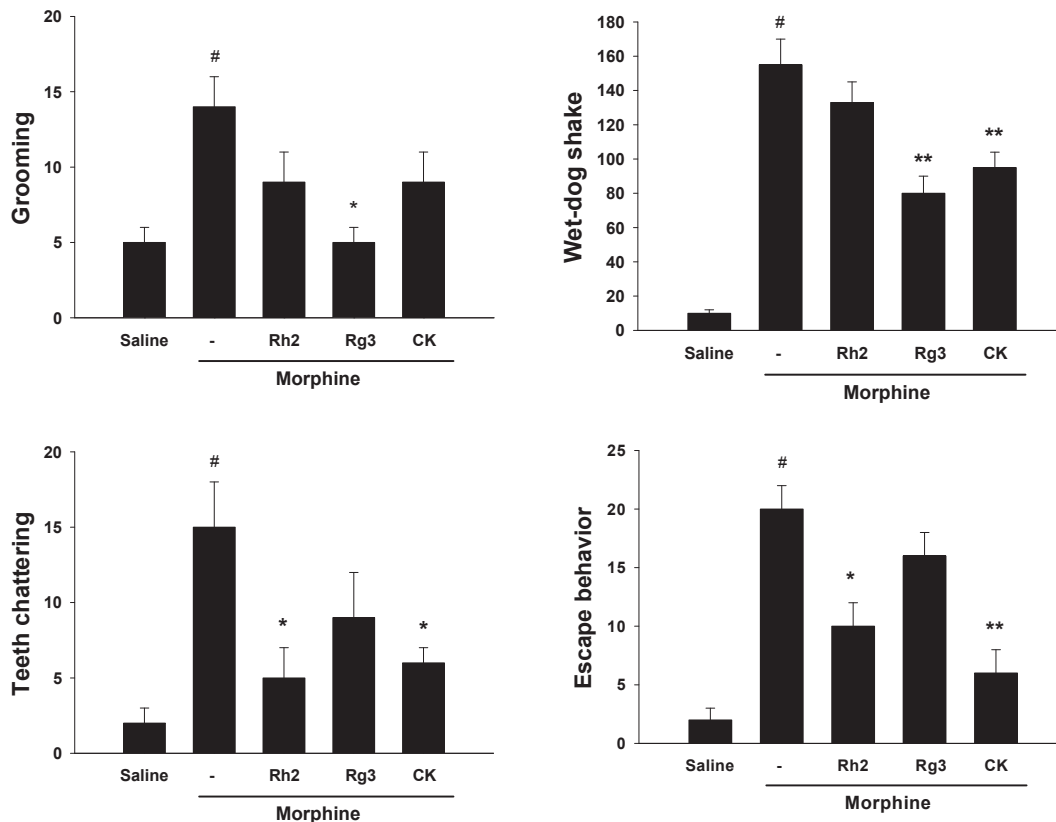
3.4. Microinfusion of ginsenosides inhibited naloxone-precipitated withdrawal symptoms in morphine-treated rats

To determine which ginsenosides were responsible for the action of RGE against morphine dependence, biotransformed ginsenosides were isolated and infused into rat brains. Ginsenosides Rh2, Rg3, and compound K isolated from RGE showed reduced behavioral patterns of morphine dependence in rats. Ginsenoside Rh2 and compound K revealed a strong effect on teeth chattering and escaping behavior of prolonged morphine-infused rats when compared with grooming and wet-dog shake (Fig. 4). In contrast, Rg3 more significantly retarded grooming and wet-dog-shake symptoms of morphine withdrawal in rats.

3.5. Effect of ginsenosides Rh2, Rg3, and compound K on phosphorylated NR1 and ERK1/2 in frontal cortical tissues of rat brain and cultured cortical neurons

Previously, it was reported that morphine induced activation of ERKs and NR1, which intricately interact with G protein-coupled μ-opioid receptors, leading to morphine-associated dependence and tolerance [7]. Therefore, we sought to evaluate if morphine induced protein expression levels of phospho-NR1 and phospho-ERK in the frontal cortex regions of rat brain, and cultured cortical cells were modulated by ginsenosides Rh2, Rg3, and compound K. We found that p-ERK protein expression was significantly reduced by the three ginsenosides in the frontal cortical regions of rat brain. While ginsenosides Rg3 and compound K significantly attenuated p-NR1 expression in the frontal cortical regions of rat brain, the effect of Rh2 was barely detected (Fig. 5). Likewise, the protein expression of p-NR1 was attenuated in cultured cortical neurons pretreated with

Fig. 4. Inhibitory effects of ginsenosides on morphine withdrawal signs in rats. Rats were infused with morphine (26 nmol/10 μL/h) and/or ginsenoside (10 μg/10 μL/h, about 600 μg/kg/d) for 7 d, and then treated with naloxone (10 mg/kg, i.p.) 6 h after the cessation of morphine infusion. Withdrawal signs were observed for 30 min after injection of naloxone. Data were expressed as mean ± SD ($n = 6$). $^{#} p < 0.05$, compared with the saline-only group. $^{*} p < 0.05$, compared with the morphine-only group. $^{**} p < 0.01$, compared with the morphine-only group. SD, standard deviation.
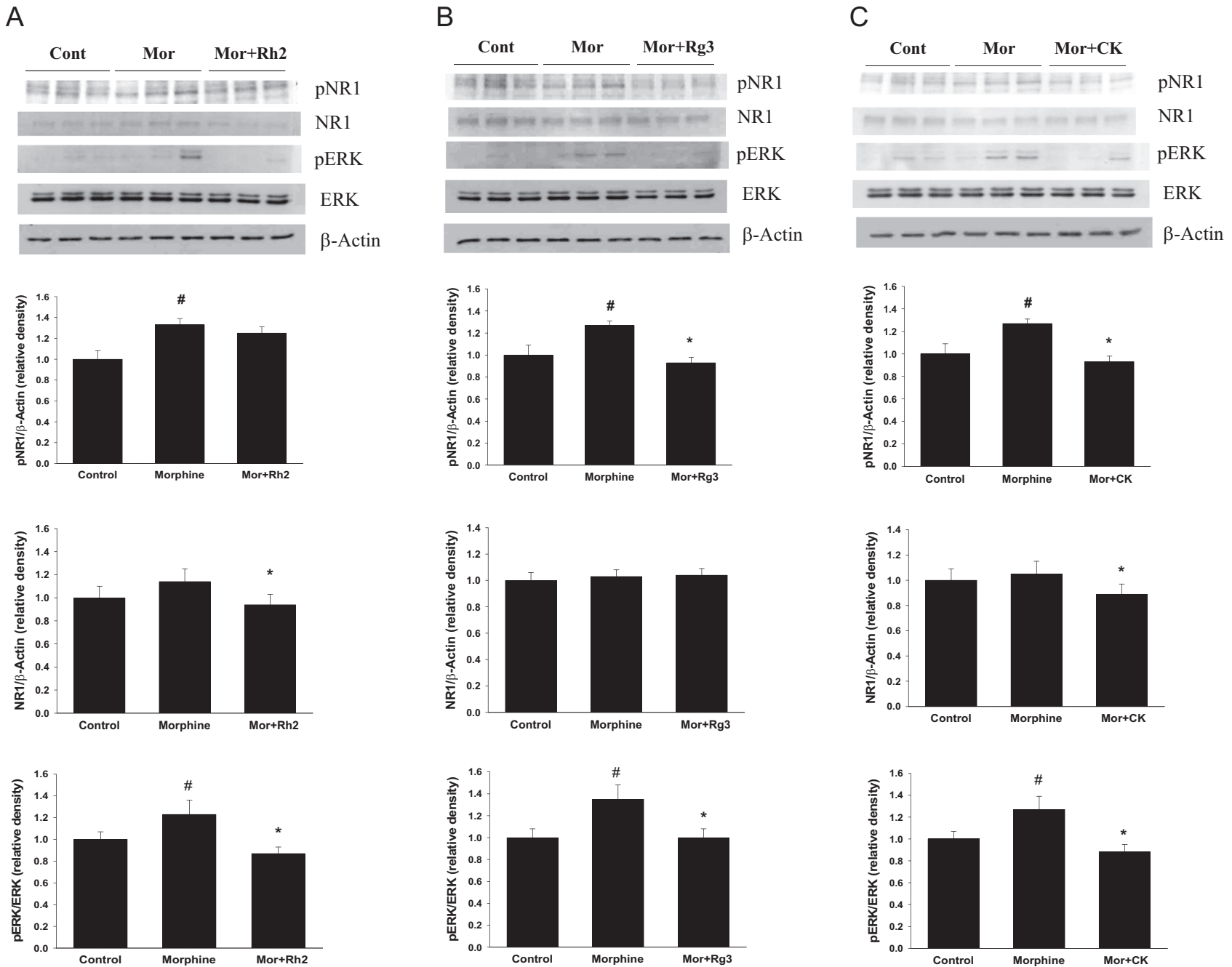
Fig. 5. Effects of ginsenosides on morphine-induced phosphorylation of NR1 and ERK in the frontal cortex region of rats. Rats were infused with morphine (26 nmol/10 μL/h) and/or ginsenoside (10 μg/10 μL/h, about 600 μg/kg/d) for 6 d. Frontal cortex region of rats were collected 8 h after the cessation of morphine infusion. Protein expressions of p-NR1, NR1, p-ERK, and ERK were examined using western blot analysis. Data were expressed as mean ± SD ($n = 6$). [#] $p < 0.05$, compared with the saline group. * $p < 0.05$, compared with morphine alone. CK, compound K; Cont, control; ERK, extracellular signal-regulated kinase; Mor, morphine; NR1, N-methyl-D-aspartate acid receptor subunit 1; SD, standard deviation.
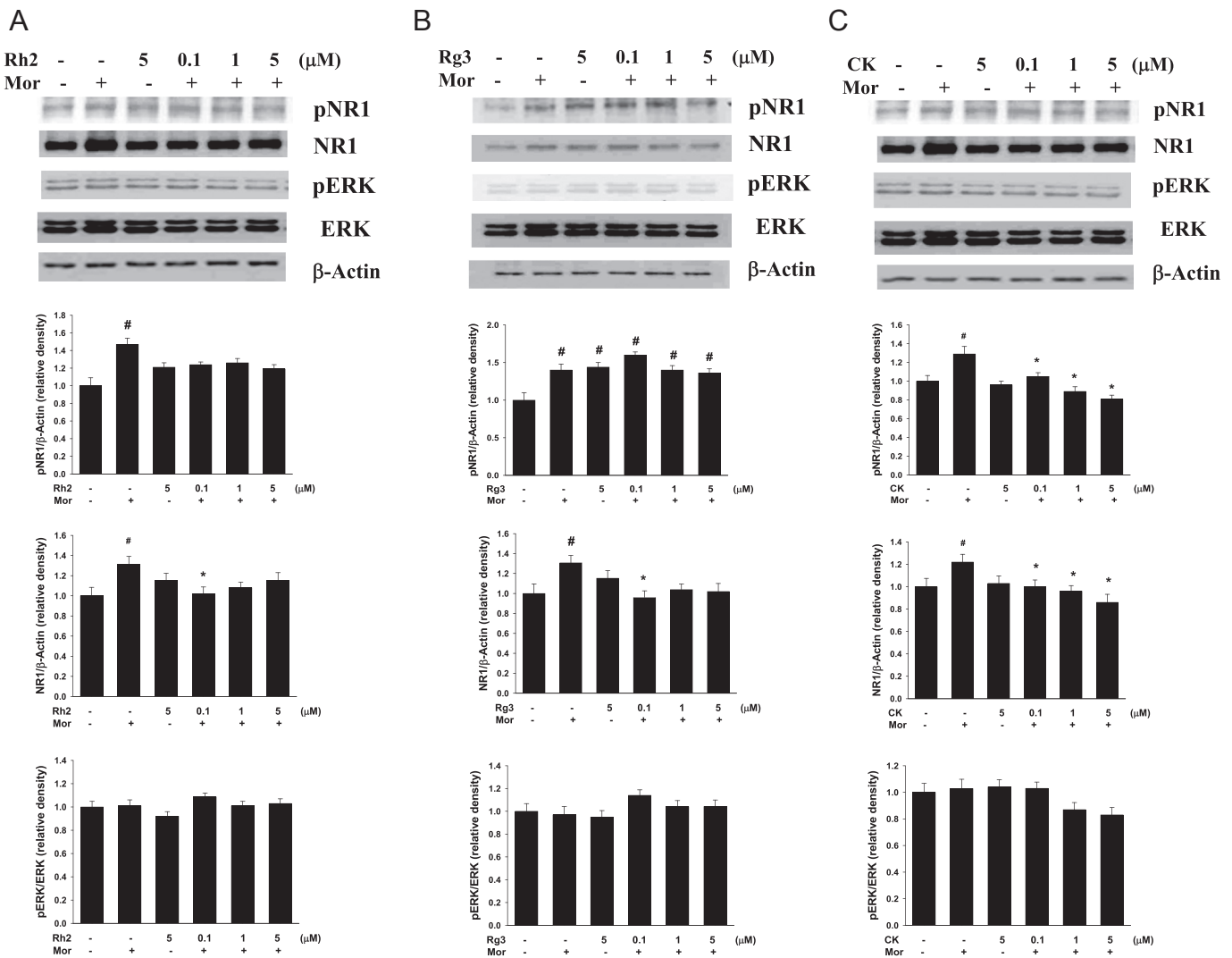
Fig. 6. Changes in protein expressions of NR1 and ERK measured in cultured cortical neurons. Cells were pretreated with ginsenosides Rh2, Rg3, and CK (0.1μM, 1μM, and 5μM, respectively) for 30 min and then exposed to morphine (1μM) for 6 h. Expressions of p-ERK and p-NR1 levels were examined using western blot analysis. All values are expressed as mean ± SD from three independent experiments. [#] $p < 0.05$, compared with the vehicle group. * $p < 0.05$, compared with morphine alone. CK, compound K; ERK, extracellular signal-regulated kinase; Mor, morphine; NR1, N-methyl-D-aspartate acid receptor subunit 1; SD, standard deviation.

ginsenosides and subjected to morphine. However, effects of ginsenosides on activated ERK during morphine treatment in cortical neurons were found to be negligible (Fig. 6).

4. Discussion

Given the importance of opioids to relieve moderate to severe pain, their unwanted effects of drug dependence, analgesic tolerance, and hyperalgesia remained an intimidating challenge. Despite all these side effects, opioids such as morphine persist as pharmacological cornerstone of modern pain therapy [26]. The interplay of different receptors and kinases involved in morphine action has paramount importance to understand the intricate nature of dependence and tolerance. Apart from the classical opioid receptors, morphine action to opioid receptor like-1 receptor, Toll-like receptor 4, and NR1 was reported for the modulation of pain and associated deleterious effects [27]. These side effects of morphine could be mitigated by plant-derived compounds such as resveratrol [11]. RGE and ginsenosides have shown neuroprotective effects [28]; however, little is known about their protective effect against morphine-induced

dependence. We found that RGE along with biotransformed ginsenosides Rh2, Rg3, and compound K ameliorated morphine-induced dependence, such as jumping and CPP in mice, and certain behavioral patterns, such as grooming, teeth chattering, wet-dog shake, and escaping, in rats (Figs. 1, 2, 4); these observations were in line with those of previous reports indicating the role of phytochemicals working against morphine withdrawal symptoms in experimental animals [3,28]. Our finding was also consistent with a previous work that shows the attenuating effect of ginger extract on morphine-induced and naloxone-precipitated withdrawal signs such as teeth chattering and jumping behavioral patterns of mice [29]. Evidence indicated that treatment with morphine is associated with the production of reactive metabolites such as morphinone that can bind cellular glutathione and reduces its bioavailability in the liver [30]. Therefore, we determined if the level of glutathione was reduced during morphine treatment and this effect was modulated by RGE. We observed that RGE restored the level of glutathione to its normal condition, and this finding was in line with that of a study revealing that suppressed glutathione level in aged mice was restored with red ginseng treatment [31].

It was reported that activation of the NMDAR—ERK signaling pathway in morphine induced CPP in rats [32]; therefore, we investigated whether ginsenoside Rh2, Rg3, or compound K modulated the protein expression of NR1 and ERK. We found that phosphorylated ERK was inhibited by the aforementioned ginsenosides in the frontal cortex tissue of rats exposed to morphine. Likewise, rats infused with ginsenosides Rg3 and compound K showed a reduced protein expression of NR1 in morphine-exposed groups when compared with Rh2, which was observed to reduce p-ERK expression moderately and the NR1 protein expression faintly. Compound K showed an excellent inhibitory effect on p-NR1 and p-ERK compared with Rg3 and Rh2, in both the frontal cortical tissues of rat brain and cultured cortical neurons. In fact, compound K has shown diverse biological properties such as anticarcinogenic, anti-inflammatory, antiallergic, antidiabetic, antiangiogenesis, anti-aging, neuroprotective, and hepatoprotective effects [33].The current discrepancies of ginsenoside actions on p-ERK or p-NR1 in the brain might be due to their differential potency to modulate cell signaling proteins; however, this warrants further investigation.

Ginsenosides alter behavioral changes by modulating neurons that seem to be associated with the GABA$_A$ receptor complex. GABA is a major inhibitory transmitter in the central nervous system. Several lines of evidence show that the GABA$_A$ receptor complex is involved in the development of opioid tolerance [15]; hence, it may be a useful target for the treatment of drug abuse and dependence [34,35]. Interestingly, the level of [^{3}H]muscimol binding in the cortex and cerebellum was increased by prolonged infusion of Rh2 and compound K [36], indicating the possibility of ginseng metabolites to modulate the GABA receptor complex and thereby ameliorating morphine dependence.

Wang et al [20] reported that Rg3 and compound K have strong anticancer effects via impeding mitogen-activated protein kinase (MAPK) signaling, which is in line with the diminished ERK phosphorylation by Rg3 and compound K noticed in the frontal cortex region of rats. It was documented that morphine induces neuro-inflammation via Toll-like receptor 4 [37]; therefore, the inhibitory effect of Rg3 or compound K on ERK phosphorylation might follow MAPK signaling downstream of Toll-like receptor 4.

Compelling evidence also indicated that opioid receptors are part of G-protein-coupled receptors, and their activation by exogenous agonists such as morphine lead to the dissociation of Gα and G$\beta\gamma$ subunits, where the Gα_i subunit inhibits cyclic adenosine monophosphate (cAMP) production and obliterates the cAMP—PKA pathway [38,39]. It is therefore intriguing to further investigate if ginsenosides intervened downstream of opioid receptors, thereby attenuating morphine dependence.

In conclusion, RGE and biotransformed ginsenosides Rh2, Rg3, and compound K reduced behavioral patterns associated with morphine dependence in mice and rats. Despite their differential effects, ginsenosides Rh2, Rg3, and compound K reduced the levels of activated NR1 and ERK proteins in the cortical regions of morphine-treated groups, suggesting that their potential action may pose against NMDAR and downstream signaling proteins.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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