Analgesic Effects of Ephedra Herb Extract, Ephedrine Alkaloids–Free Ephedra Herb Extract, Ephedrine, and Pseudoephedrine on Formalin-Induced Pain

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INTRODUCTION

The analgesic effect of Ephedra Herb (EH) is believed to be derived from the anti-inflammatory action of pseudoephedrine (Pse). We recently reported that ephedrine alkaloids–free EH extract (EFE) attenuates formalin-induced pain to the same level as that achieved by EH extract (EHE), which suggests that the analgesic effect of EH may not be due to ephedrine alkaloids (EAs). To examine the contribution of EAs to the analgesic effect of EH, mice were injected with formalin to induce a biphasic pain reaction (first phase, 0–5 min; second phase, 10–45 min) at various time points after oral administration of the following test drugs: ephedrine (Eph), Pse, “authentic” EHE from Tsumura & Co. (EHE-Ts), EFE, and EHE that was used as the source of EFE (EHE-To). Biphasic pain was suppressed at 30 min after administration of Eph, EHE-Ts, and EHE-To. At 6 h after administration of EFE, EHE-To, and Pse—and at 4 to 6 h after administration of EHE-Ts—only second-phase pain was suppressed; however, the effect of Pse at 6 h was not significant. These results suggested that EHE has a biphasic analgesic effect against biphasic formalin-induced pain: in the first phase of analgesia (30 min after administration), biphasic pain is suppressed by Eph; in the second phase of analgesia (4–6 h after administration), second-phase pain is alleviated by constituents other than EAs, although Pse may partially contribute to the relief of second-phase pain.

Key words: Ephedra Herb; ephedrine; analgesic effect; formalin test; pain drug

The analgesic actions of EH have been attributed to the anti-inflammatory effect of Pse, which inhibits the biosynthesis of prostaglandin E2. However, we previously reported that repeated oral administration of EFE attenuates formalin-induced pain to the same levels as that achieved by administration of EHE. In addition, we recently reported that EHE may suppress capsaicin-induced pain by desensitizing transient receptor potential vanilloid 1 (TRPV1), and that Eph has no effect on TRPV1. These findings suggest that the analgesic effects of EHE may not be due to EAs.

The formalin test is widely used for evaluating analgesic effects. Formalin induces biphasic behavioral-related pain: immediate pain (first phase) and late-onset pain (second phase). Pain in the first phase is thought to be induced by direct stimulation of nociceptors such as transient receptor potential ankyrin 1 (TRPA1), and pain in the second phase is the result of tonic afferent input from peripheral nerves involved in inflammatory mechanisms, and central sensitization at the spinal cord level. Compounds that typically suppress both phases are opioids such as morphine, and transient receptor potential ankyrin 1 antagonists such as HC-030031. Compounds that suppress the second phase include nonsteroidal anti-inflammatory drugs (NSAIDs), gabapentin, and N-methyl-D-aspartate antagonists and anti-tropomyosin receptor kinase A (TrkA) monoclonal antibodies such as MNAC13. Therefore, the formalin test is useful for determining analgesic effects.
In this study, we used the formalin test to evaluate the analgesic effects of EHE, EFE, Eph, and Pse in order to clarify whether EAs contribute to the analgesic effect of EH.

MATERIALS AND METHODS

Materials EHE (Lot. 2091037010) was purchased from Tsumura & Co. (Tokyo, Japan). For the purpose of this study, EHE from Tsumura & Co. was deemed “authentic,” because of the consistent quality of EHE maintained by the company. Hereafter, EHE from Tsumura & Co. will be referred to as EHE-Ts. A three-dimensional HPLC profile of EHE-Ts is shown in Supplementary Fig. 1A.

The preparation of EFE was described in detail in our previous report. Briefly, EH (10 kg) was added to water (100 L); extraction was performed at 95°C for 1 h. One portion of the extract was subjected to ion exchange to remove EAs, after which that portion was lyophilized to form EFE. The remaining portion of the extract was lyophilized without removal of EAs; and that lyophilized powder, as the starting material for the production of EFE, will be hereafter be referred to as EHE-To. The yield of EFE was about 84%, and the content of EAs in EFE was undetectable level (<0.05 ppm). Three-dimensional HPLC profiles of EHE-To and EFE are shown in Supplementary Figs. 1B and C, respectively; the concentrations of Eph and Pse in EHE-To as quantified by HPLC were approximately 4% for Eph and 1.6% for Pse. EHE-To was used as a comparison for evaluating the analgesic effect of EFE.

Eph chloride was purchased from Nichi-Iko Pharmaceutical Co., Ltd. (Toyama, Japan). Pse was provided by Alps Pharmaceutical Ind. Co., Ltd. (Gifu, Japan). Experiments on Eph and Pse were performed by a “stimulants raw materials handler,” as defined by Japanese law.

Diclofenac sodium and formaldehyde solution were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Animals Specific pathogen-free ddY mice (5 weeks old, male) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Prior to the experiments, the mice were acclimatized for one week at a temperature of 25 ± 2°C, humidity of 50 ± 10%, and a 12 h light/12 h dark cycle. All animal experiments were performed between 10:00 a.m. and 6:00 p.m.

The protocol for animal experiments was approved by the Institutional Animal Care and Use Committee of Kitasato University. Experiments were performed in accordance with the Kitasato University guidelines for animal care, handling, and termination, which are in line with international and Japanese guidelines for animal care and welfare.

Formalin-Induced Paw Licking Test Mice were individually placed in transparent acrylic cylinder cages (height of 200 mm; diameter of 100 mm) for adaptation. After 20 min, a microsyringe (MS-NG50; Ito Microsyringe Co., Ltd., Tokyo, Japan) with a sharp needle (28 G; Ito Microsyringe, Co., Ltd.) was used to inject 10 µL of saline solution containing 2.5% formalin into the plantar surfaces of the mice’s left hind paws. Licking behavior was recorded with a digital video camera for a period of 45 min. In this study, formalin induced biphasic behavioral-related pain was defined as first phase (0–5 min) and second phase (10–45 min). And the analgesic effects among EHE-Ts, EHE-To, EFE, Eph, and Pse were compared for each phase.

To investigate the time-dependent effect of EHE-Ts on formalin-induced pain, the mice were grouped into 16 groups. They were orally administered water (vehicle) or 700 mg/kg of EHE-Ts. After a period of 0, 0.5, 1, 2, 4, 6, 8, and 24 h, 2.5% formalin (10 µL) was injected into the plantar surfaces of their left hind paws. Licking time was measured for 45 min after each formalin injection.

To investigate the dose-dependent effect of EHE-To and EFE on formalin-induced pain, the mice were grouped into 7 groups. They were orally administered water (vehicle), 175 to 700 mg/kg of EHE-To, or 175 to 700 mg/kg EFE. Thirty minutes and 6 h later, 2.5% formalin was injected as described above.

To investigate the effects of alkaloidal components of EHE-To (i.e., Eph and Pse) against formalin-induced pain, the mice were grouped into 5 groups. They were orally administered water (vehicle), EHE-To (700 mg/kg), EFE (700 mg/kg), Eph (30 mg/kg), or Pse (12 mg/kg). The dosages of Eph and Pse were based on their content in EHE-To. Thirty minutes and 6 h after administration of the test drugs, 2.5% formalin was injected as described above.

Rotarod Test In this study, we used a rotarod treadmill (MK-600; Muromachi Kikai Co., Ltd., Tokyo, Japan) to assess the physical performance of the mice. The rotarod test was performed as previously reported. 19 The day prior to the test, the mice were familiarized with the rotarod test by placing them on a rotating rod (28 rpm) for 5 min; this training was repeated 6 times every hour. On the day of the rotarod test, the mice were trained again; those that fell off the rotating rod were excluded from this experiment. The mice were orally administered water (vehicle), EHE-To (700 mg/kg), EFE (700 mg/kg), Eph (30 mg/kg), or Pse (12 mg/kg). We measured their endurance time (the length of time the mice could remain on the rotarod) at 30 min and 6 h.

Statistical Analysis All data were analyzed by ANOVA, and are expressed as mean ± standard error of the mean. Significant differences between the control and treatment groups were determined by Dunnett’s multiple comparison test or Student’s t-test. All statistical analyses were performed using Prism 7 (GraphPad Software Inc., San Diego, CA, U.S.A.). Statistical significance was determined based on values of p < 0.05.

RESULTS

Analgesic Effect of Orally Administered EHE-Ts against Formalin-Induced Pain We used the analgesic effect of diclofenac sodium to evaluate the reliability of the paw licking test for assessing formalin-induced pain. Orally administered diclofenac sodium significantly reduced second-phase paw licking time, but had no effect on first-phase paw licking time (Supplementary Fig. 2). Nonsteroidal anti-inflammatory drugs such as diclofenac sodium are known to suppress only second-phase pain. Thus, the above results indicated that our paw licking test was effective for measuring formalin-induced pain.

Next, we assessed the analgesic effects of single oral administrations of EHE-Ts. We utilized EHE-Ts in this experiment because it was “authentic” EHE, such that its effects reflected the analgesic effects of EHE in general. The dose
(700 mg/kg) of EHE-Ts was the same as in that used in our previous study. The analgesic effects of EHE-Ts against formalin-induced pain were examined at different time points from 0 to 24 h after administration of EHE-Ts (Fig. 1). Second-phase paw licking times significantly declined 0 to 30 min, and 4 to 6 h after administration of EHE-Ts (Fig. 1). Thus, both first-phase and second-phase pain were transiently suppressed at 30 min after oral administration of EHE-Ts, but only second-phase pain was persistently suppressed 4 to 6 h after oral administration of EHE-Ts. This suggested that EHE-Ts possesses biphasic analgesic effects: a rapid and transient effect; and a slow, continuous effect.

**Comparison of Analgesic Effects at 30 min and 6 h after**
Oral Administration of EHE-To and EFE  To elucidate whether EAs play an important role in the biphasic analgesic effects of EHE, we compared the effect of EFE to that of EHE-To at 30 min and 6 h after oral administration.

Thirty minutes after administration, EHE-To reduced both first- and second-phase paw licking times in a dose-dependent manner. EFE reduced second-phase paw licking time in a dose-dependent manner (Fig. 2), but not first-phase paw licking time.

Six hours after administration, both EHE-To and EFE reduced second-phase paw licking time in a dose-dependent manner (Fig. 3), but not first-phase paw licking time.

These results suggested that EAs mainly contribute to the reduction of first-phase paw licking times at 30 min after administration of EHE-To.

Comparison of Effects at 30 min and 6 h after Oral Administration of EHE-To, EFE, Eph, and Pse  We examined the effects of the major EAs (i.e., Eph and Pse) against formalin-induced pain at 30 min and 6 h after oral administration. The doses of Eph and Pse were based on their content in 700 mg/kg doses of EHE-To.

Thirty minutes after administration, EHE-To and Eph significantly suppressed both first- and second-phase pain; and although EFE and Pse suppressed second-phase pain (Fig. 4A), the effects were not significant. These results suggested that Eph plays an important role in the suppression of both first- and second-phase pain at 30 min after administration of EHE-To.

Six hours after administration, EHE-To, EFE, Eph, and Pse...
had no effect on first-phase pain (Fig. 4B). EHE-To and EFE significantly suppressed second-phase pain; and although Pse suppressed second-phase pain (Fig. 4B), the effect was not significant. Interestingly, Eph had almost no effect on second-phase pain (Fig. 4B). These results suggest that during the second phase of EHE-To-induced analgesia, the primary contributors to the relief of second-phase pain were non-alkaloid constituents, although Pse may have partially contributed to relief of second-phase pain.

The Effects of EHE-To, EFE, Eph, and Pse on Physical Performance In order to confirm that reduction in paw licking was not caused by physical disability, the physical performance of mice on a rotarod treadmill at 30 min and 6 h after oral administration of EHE-To, EFE, Eph, and Pse was compared to that of mice from the vehicle group. There were no significant differences in physical performance between the test groups and vehicle group (Fig. 5). These results indicate that oral administration of EHE-To, EFE, Eph, and Pse had no effect on physical performance.

DISCUSSION

We previously reported that in mice, oral administration of 350–700 mg/kg/d of EHE-To or EFE for 3 d significantly alleviates formalin-induced second-phase pain in a dose-dependent manner at 6 h after the final dose of either test drug. 3) In this study, we assessed the analgesic action of a single dose of EHE-Ts against formalin-induced pain with respect to time. At 30 min after administration, EHE-Ts suppressed both first and second-phase pain; but at 4–6 h after administration, EHE-Ts reduced only second-phase pain. This study is the first to report on the biphasic analgesic effect of EHE: a rapid transient effect, and slow continuous effect.

At 30 min after oral administration, EHE-Ts, EHE-To, and Eph suppressed both first and second-phase pain; but EFE and Pse did not exert such effects. Therefore, it is possible that during the first phase of analgesia, the suppression of biphasic pain by EHE was attributable to Eph. Our results are supported by Wei et al., who reported that the concentration of Eph in rat serum peaks at 30 min after oral administration of EHE. 20)

We believe the mechanism for the analgesic effects of Eph is as follows: It is known that nociceptive stimulation is regulated by endogenous analgesic systems such as the descending noradrenergic pathway. 21) In the descending noradrenergic pathway, noradrenaline-containing nuclei terminals project into the spinal cord and release noradrenalin from neuron terminals to suppress pain. Noradrenaline inhibits the release of glutamate from presynaptic sites by activating α2A-adrenoceptors, and promotes the release of γ-aminobutyric acid and glycine from inhibitory interneurons by activating α1A-adrenoceptors. Furthermore, noradrenaline directly activates α2A-adrenoceptors on postsynaptic neurons to suppress the excitation of postsynaptic neurons. 22–23) It is believed that Eph and its analogues stimulate the central and autonomic systems by mimicking the action of adrenaline through direct stimulation of adrenergic receptors, and through indirect stimulation by inducing the release of noradrenaline from axon terminals. 24–25) Therefore, Eph might suppress formalin-induced pain by affecting the central nervous system in a manner similar to that of the α2-adrenoceptor agonist dexmedetomidine, which induces analgesic effects via stimulation of α2-adrenoceptors. 26)

At 6 h after oral administration, EHE-Ts, EHE-To, and EFE suppressed second-phase pain; but Eph did not exert that effect. Therefore, it is possible that during the second phase of analgesia, the suppression of second-phase pain by EHE is attributable to constituents other than EAs. Studies on other constituents have been performed by researchers such as Wang et al., who reported that polysaccharides isolated from *Ephedra sinica* have immunosuppressive activity and are effective in the treatment of rheumatoid arthritis. 27) In addition, EH is known to contain various flavonoids, such as herbacetin-glycosides and tannins. 28–30) We previously reported the isolation of herbacetin 7-O-glycosides and herbacetin 7-O-neohesperidoside from EHE. 28) Herbacetin, an aglycon of those herbacetin glycosides, mitigates formalin-induced second-phase pain by inhibiting nerve growth factor (NGF)-TrkA signals related to inflammatory responses. 31,32) Finally, we previously reported that EHE alleviates capsaicin-induced pain by desensitizing TRPV1 on peripheral sensory nerves, and that Eph has no effect on TRPV1; 10) which indicates that EHE and EFE may induce analgesic effects via modulation of NGF-TrkA and TRPV1 in peripheral sensory nerves.

The delayed analgesic effect of constituents other than EAs against formalin-induced second-phase pain is thought to be caused by the slow absorption of such constituents from the gastrointestinal tract. For instance, the absorption and metabolism of flavonoids by the gastrointestinal tract are mediated by various pathways, 33) such that the time to peak concentration ranges from 0.5 to 20 h. 34) At 30 min after oral administration of EFE, the serum concentrations of non-alkaloid constituents may not have been sufficient enough for the full expression of analgesic effects or may be unstable. Therefore, there were also discrepancy between results in Figs. 2 and 4A on analgesic effect of EFE.

Kasahara et al. suggested that Pse induces anti-inflammatory effects by inhibiting the biosynthesis of prostaglandin E2. 29) Therefore, the analgesic effect of EH is believed to result...
from the anti-inflammatory action of Pse. However, the doses of Pse in the report by Kasahara et al. ranged from 50 to 200 mg/kg on ddY mice,9 which were 4 to 16 times higher than the amount of Pse used in this report. We administered 12 mg/kg Pse because the concentration of Pse in EHE-To was 1.6%. The fact that Pse showed a tendency to suppress second-phase pain after 6 h (Fig. 4B) suggested that it contributes in part to the analgesic effects of EHE-To, but the low Pse content in EH makes it difficult for the analgesic effect of EH to be explained by the inflammatory action of Pse alone.

In summary, our study revealed that both Eph and non-EA constituents contribute to the analgesic effect of EH. Moreover, we demonstrated that a 700 mg/kg dose of either EHE-To or EFE can significantly suppress formalin-induced second-phase pain. Because 700 mg/kg EFE does not induce the excitation, insomniania, and arrhythmias associated with 700 mg/kg EHE-To, EFE has great potential as a safe and effective analgesic drug for the treatment of arthritic conditions such as osteoarthritis in the elderly. Thus, we are currently evaluating the efficacy of EFE against arthralgia in an arthritis mouse model, and we are attempting to isolate the active constituents of EFE in order to analyze their structures.

Acknowledgments This research was supported by Grants-in-Aid from the Japan Health Sciences Foundation (public-private sector joint research on publicly essential drugs), the Research on Development of New Drugs from the Japan Agency for Medical Research and Development, and the All Kitasato Project Study Collaborative Research.

Conflict of Interest Oriental Medicine Research Center of Kitasato University received donations from Tsumura Co., Ltd. Furthermore, Kitasato University, the National Institute of Health Sciences, Matsuyama University, Tokiwa Pharmaceutical Co., Ltd., and Zeria Pharmaceutical Co., Ltd. are co-applicants for the relevant patent.

Supplementary Materials The online version of this article contains supplementary materials.

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