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The Analgesic Effects and Mechanisms of Orally Administered Eugenol

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In the present study, the antinociceptive profiles of eugenol were examined in ICR mice. Eugenol administered orally (from 1 to 10 mg/kg) showed an antinociceptive effect in a dose-dependent manner as measured in the acetic acid-induced writhing test. Duration of antinociceptive action of eugenol maintained at least for 30 min. Moreover, the cumulative response time of nociceptive behaviors induced by an intraplantar formalin injection was reduced by eugenol treatment during the 2nd phases. Furthermore, the cumulative nociceptive response time for intrathecal injection of substance P (0.7 μg) or glutamate (20 μg) was diminished by eugenol. Intraperitoneal pretreatment with yohimbine (α2-adrenergic receptor antagonist) or naloxone (opioid receptor antagonist) attenuated antinociceptive effect induced by eugenol in the writhing test. However, methysergide (5-HT serotonergic receptor antagonist) did not affect antinociception induced by eugenol in the writhing test. Our results suggest that eugenol shows an antinociceptive property in various pain models. Furthermore, this antinociceptive effect of eugenol may be mediated by α2-adrenergic and opioidergic receptors, but not serotonergic receptor.

Key words: Caffeic acid, Antinociception, Inflammatory pain, Opioid receptor, α2-Adrenergic receptor

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INTRODUCTION

Eugenol is an aromatic molecule found in several plants such as clove, bay leaves and allspice, and has been used as a topical treatment for pain in phenolic dental procedures (Ohkubo and Shibata 1997; Kim et al., 2003). The agent has sedative and anodyne effects, but concomitantly shows an irritant action (Sneddon and Glew, 1973). It also has antioxidant, anti-inflammatory and antiseptic properties (Li et al., 2006). The analgesic action of eugenol had long been attributed to its action as a non-specific counter-irritant. In addition, it has been reported that the agent inhibits sensory nerve activity (Ozeki, 1975; Trowbridge et al., 1982), or has a potent inhibitory action on PGI_2 production (Hirafuji, 1984). In nervous system, eugenol is neuroprotective against excitotoxicity, ischemia and amyloid-peptide (Wie et al., 1997; Won et al., 1998; Irie and Keung, 2003), inhibits the conduction of action potential in sciatic nerves (Kozam, 1977), and improves neuronal and vascular complications in experimental diabetes (Nangle et al., 2006). Eugenol suppresses epileptiform field potentials and spreading depression in hippocampus and neocortex, which indicate the potential for eugenol to use in the treatment of epilepsy and cephalic pain (Müller et al., 2006). On the other hand, it has also been reported that eugenol possesses a capsaicin-like action on peripheral endings of primary afferents of the rat urinary bladder (Patacchini et al., 1990). Eugenol may exert their antinociceptive effects via the capsaicin receptor located on sensory terminals in the spinal cord (Ohkubo and Shibata, 1997).

However, further antinociceptive profiles and the antinociceptive mechanism of eugenol has not been well characterized. Thus, we, in the current study,

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attempted to characterize antinociceptive profiles and mechanisms of eugenol in several pain models.

MATERIALS AND METHODS

These experiments were approved by the University of Hallym Animal Care and Use Committee (Registration Number: Hallym 2009-05-01). All procedures were conducted in accordance with the 'Guide for Care and Use of Laboratory Animals' published by the National Institutes of Health and the ethical guidelines of the International Association for the Study of Pain.

Experimental animals

Male ICR mice (MJ Co.) weighing 20-25 g were used for all the experiments. Animals were housed 5 per cage in a room maintained at 22 ± 0.5°C with an alternating 12 h light-dark cycle. Food and water were available *ad libitum*. The animals were allowed to adapt to the laboratory for at least 2 h before testing and were only used once. Experiments were performed during the light phase of the cycle (10:00-17:00).

Oral administration, and intraperitoneal and intrathecal injections

Oral administration was performed with gage in a volume of 500 μL/25 g body weight. Intraperitoneal (*i.p.*) injection was conducted to unanesthesized mice with volume of 250 μL. The intrathecal (*i.t.*) administration was performed following the method of Hylden and Wilcox (Hylden and Wilcox, 1980, 1981) using a 30-gauge needle connected to a 25 μL Hamilton syringe with polyethylene tubing. The *i.t.* injection volume was 5 μL and the injection site was verified by injecting a similar volume of 1% methylene blue solution and determining the distribution of the injected dye in the spinal cord. The dye injected *i.t.* was distributed both rostrally and caudally but with short distance (about 0.5 cm from the injection site) and no dye was found visually in the brain. The success rate for the injections was consistently found to be over 95%, before the experiments were done.

Acetic acid-induced writhing and intraplantar formalin tests

For the writhing test (Koster et al., 1959), 1% acetic acid was injection *i.p.* and then, the animal were immediately placed in an acrylic observation chamber (20 cm high, 20 cm diameter). The number of writhes was counted during 30 min after the injection of acetic acid. A writhe was defined as a contraction of the abdominal muscles accompanied by an extension of the forelimbs and elongation of the body. For the formalin

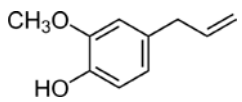

Fig. 1. Structure of eugenol.

test (Hunskaar et al., 1985), 10 μL of 5% formalin was injected subcutaneously under the plantar surface of the left hindpaw. Following injection of formalin, the animals were immediately placed in an acrylic observation chamber, and the time spent licking, shaking and biting the injected paw was measured with a stop-watch timer and considered as indication of nociception. The early phase of the nociceptive response normally peaked 0 to 5 min, and the last phase 20 to 40 min after formalin injection, representing the direct effect on nociceptors and inflammatory nociceptive responses, respectively (Hunskaar and Hole, 1987). Animals were pretreated orally once with vehicle (control) or eugenol at various doses (from 1 to 10 mg/kg) 30 min prior to performing the acetic acid-induced writhing and formalin tests.

Substance P- or glutamate induced nociceptive behavioral test

Vehicle (control) or 10 mg/kg of eugenol was pretreated orally 30 min prior to performing *i.t.* injection of substance P (0.7 μg/5 μL) or glutamate (20 μg/5 μL). Immediately after *i.t.* injection with substance P or glutamate the mice were placed in an observation chamber (20 cm high, 20 cm diameter) and their nociceptive behavioral responses were recorded during 30 min. The cumulative response time of licking, scratching and biting episodes directed toward the lumbar and caudal region of spinal cord were measured with a stop-watch timer (Hylden and Wilcox, 1981).

Pretreatment of antagonists

At first, mice were pretreated *i.p.* with either saline, yohimbine (5 mg/kg), methysergide (5 mg/kg), or naloxone (5 mg/kg) 10 min before oral administration of vehicle as a control or a fixed dose of eugenol (10 mg/kg). And then, the writhing response was tested 30 min after the treatment with either vehicle or eugenol (Suh et al., 1996, 1997, 1999; Choi et al., 2003; Takashi et al., 2003; Park et al., 2009).

Drugs

All drugs were purchased from Sigma Chemical Co. Naloxone, yohimbine, methysergide were dissolved in saline. eugenol was prepared following steps: (A) 1 g of eugenol was dissolved in 0.5 mL of ethanol plus 0.5 mL of polyethylene glycol 400. (B) Separately, 100 mg

of sodium carboxymethylcellulose was dissolved in 9 mL of distilled water. (C) Finally, Solution (A) and Solution (B) were vigorously mixed. This solution excluding eugenol was used as vehicle control. All drugs were prepared just before use.

Statistical analysis

Data were presented as the mean ± S.E.M. The statistical significance of differences between groups was assessed with one-way ANOVA with Bonferroni's post-hoc test using GraphPad Prism version 4.0 for Windows XP (GraphPad Software); $p < 0.05$ was considered significant.

RESULTS

Effect of eugenol on the nociceptive behavior induced by acetic acid, formalin, substance P and glutamate

Eugenol attenuated the acetic acid-induced writhing numbers in a dose-dependent manner (Fig. 2A). Treatment with eugenol at the dose of 10 mg/kg led to 65% decrease in the acetic acid-induced writhing response compare to the control group of mice. In addition, the time-course study showed that pretreatment with eugenol for 30 min attenuated the acetic acid-induced writhing response compare to the control group of mice (Fig. 3). However, pretreatment with eugenol for 60 or 120 min did not affect acetic acid-induced writhing response (Fig. 3). In vehicle-treated control group, injection of 5% formalin caused acute, immediate nociceptive formalin responses (*i.e.*, licking/flinching and biting the injected paw) that lasted for 5 min (1st phase response). The 2nd phase nociceptive responses began about 20 min after formalin administration and lasted for about 20 min (20-40 min after formalin injection). In eugenol-treated mice, the nociceptive behaviors induced by intraplantar injection of formalin were decreased as compared with control group of mice during the only 2nd phases (Fig. 2B). Treatment

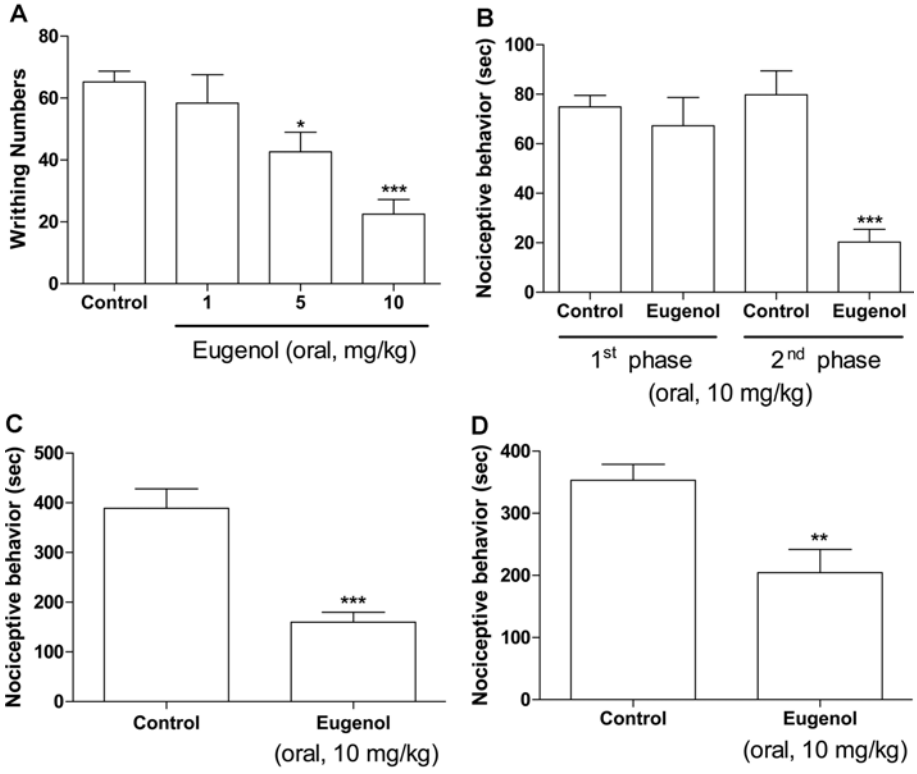

Fig. 2. Effect of eugenol on the nociceptive response induced by various pain models. Various does (from 1 to 10 mg/kg) of eugenol were administered orally and then, 0.25 mL of 1% acetic acid solution was injected intraperitoneally 30 min after treatment. The number of writhing was counted for 30 min following acetic acid injection (**A**). Animal were pretreated orally with eugenol (10 mg/kg) for 30 min prior to the formalin (5%, 10 μL) injection subcutaneously into the plantar aspect of the left side hindpaw. The cumulative response time of licking, biting and shaking the injected paw was measured during the period of 0-5 min (1st phase) and 20-40 min (2nd phase) (**B**). Eugenol (10 mg/kg) was administered orally for 30 min prior to the substance P (**C**; 0.7 μg per 5 μL) or glutamate (**D**; 20 μg per 5 μL) injection intrathecally. The cumulative response time of licking, scratching and biting episodes was measured for 30 min. The vertical bars indicate the standard error of the mean. The number of animal used for each group was 8-10 (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with control group).

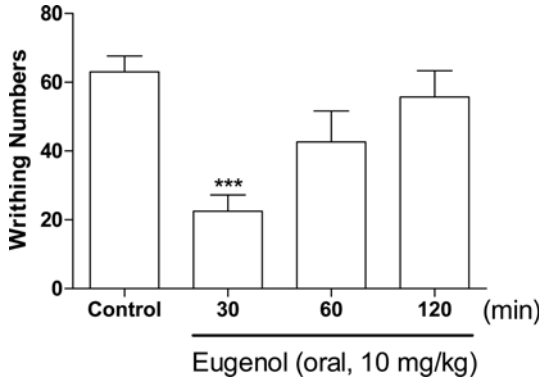

Fig. 3. Effect of eugenol on the acetic acid-induced writhing response in time course. Eugenol (10 mg/kg) was administered orally and then, 0.25 mL of 1% acetic acid solution was injected intraperitoneally 30, 60 and 120 min after treatment. The number of writhing was counted for 30 min following acetic acid injection. The vertical bars indicate the standard error of the mean. The number of animal used for each group was 8-10 (***$p < 0.001$, compared with control group).

with eugenol at the dose of 10 mg/kg did not affected in the 1st phase of formalin test over the control group of mice. However, the effect of eugenol led to 74% decrease in the 2nd phase of formalin test over the control group of mice. In vehicle-treated control mice, *i.t.* injection of substance P (0.7 μg) or glutamate (20 μg) caused acute, immediate behavioral responses, *i.e.*, licking, scratching and biting the lumbar or caudal region, which lasted about 30 min. As shown in Fig. 2C, cumulative nociceptive response times for *i.t.* administration of substance P was significantly diminished by 61%. As shown in Fig. 2D, cumulative nociceptive response times for *i.t.* administration of glutamate was significantly diminished by 50%.

Effect of opioidergic, serotonergic and adrenergic system on the inhibition of writhing response induced by Aster koraiensis extract

We examined the possible involvement of opioidergic, serotonergic and adrenergic system in the eugenol-induced antinociception. The pretreatment with methysergide (serotonergic receptor antagonist, Fig. 4B) did not affect eugenol-induced antinociception. However, the blockade of opioidergic receptor with systemic pre-administration of naloxone (Fig. 4A) and α_2-adrenergic receptor with systemic pre-administration of yohimbine (Fig. 4C) abolished the eugenol-induced inhibition of the writhing response. The treatment of naloxone, methysergide or yohimbine itself did not affect the writhing response (Fig. 4).

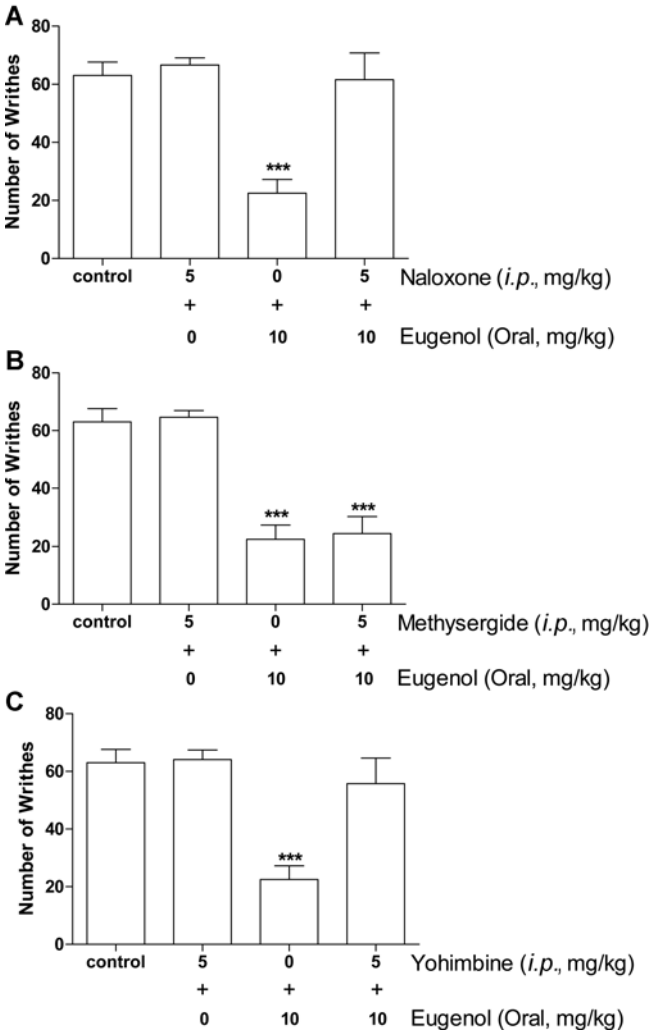

Fig. 4. Effect of naloxone (**A**), methysergide (**B**) and yohimbine (**C**) injected intraperitoneally (*i.p.*) on inhibition of the writhing response induced by eugenol administered orally. Naloxone (5 mg/kg), Methysergide (5 mg/kg) or yohimbine (5 mg/kg) was pretreated intraperitoneally for 10 min, before oral administration of vehicle or eugenol (10 mg/kg). Eugenol or vehicle was administered orally and then, 0.25 mL of 1% acetic acid solution was injected *i.p.* 30 min after treatment. The number of writhing was counted for 30 min following acetic acid injection. The vertical bars denote the standard error of the mean. The number of animal used for each group was 8-10 (***$p < 0.001$, compared with control group).

DISCUSSION

In the present study, we found that eugenol administered orally produces antinociception in various pain models. We examined the effect of eugenol on the acetic acid-induced writhing and intraplantar formalin test. Intraperitoneal (*i.p.*) injection of acetic acid can produce the peritoneal inflammation (acute peritonitis), which cause a response characterized by contraction of the

abdominal muscles accompanying an extension of the forelimbs and elongation of the body.

This writhing response is considered as a visceral inflammatory pain model (Koster et al., 1959, for review, see Vyklicky, 1979). The eugenol treatment diminished the number of acetic-acid-induced visceral nociception, which was effected at 30 min after the treatment. *I.p.* administration of dilute acetic-acid produces a characteristic writhing response in mouse. This behavior is considered to be evidence of peritoneovisceral pain, since acetic-acid directly activates visceral and somatic nociceptors innervating the peritoneum and induces inflammation not only in subdiaphragmatic visceral organs, but also in subcutaneous muscle walls (Satyanarayana et al., 2004). There is evidence that polymodal C fibers and Ad fibers are present in the gut (Cervero and Laird, 1999; Satyanarayana et al., 2004). Acetic-acid causes tissue damage and releases pain-producing substances that activate nociceptors on the sensory nerve fibers (Ulugol et al., 2006). In the present study, we clearly showed the antinociceptive effect of eugenol in an acetic acid-induced writhing test.

Moreover, in the formalin test, we showed that eugenol had an antinociceptive effect in a dose-dependent manner during the only 2nd phase. It is widely agreed that the nociceptive behaviors manifested during the acute 1st phase may be caused by the direct effect on peripheral nociceptors activating primary afferent fiber. It is followed by the tonic 2nd phase, which may be resulted from the tonic inflammatory nociceptive response. Numerous studies have reported that peripherally acting drugs such as aspirin and glucocorticoid only inhibit the 2nd phase in the formalin test (Hunskaar et al., 1985; Hunskaar and Hole, 1987; Puig and Sorkin, 1989; Choi et al., 2001; Chung et al., 2001). In contrast, aminipyrine and mefenamic acid, which act on both central and peripheral sites, inhibit nociceptive behaviors manifested during both phases. Therefore, eugenol may be, at least, a centrally acting compound, because oral treatment with eugenol inhibited the only 2nd phase of formalin test. Furthermore, it has been reported that *i.t.* injection of substance P or glutamate in mice can also elicit nociceptive responses, consisting of biting, scratching and licking the caudal parts of the body (Hylden and Wilcox, 1981; Cumberbatch et al., 1994). We found in the present study that eugenol was also effective in attenuating substance P- or glutamate-induced nociceptive responses. These results suggest furthermore that eugenol may exert their antinociceptive effect via the central sites, possibly spinally mediated mechanisms.

The roles of opioid, serotonergic and adrenergic receptors in the regulation of modulation of nociceptive processing have been demonstrated in many previous studies. For example, it is well known that opioid receptors are involved in the antinociception (Schmauss and Yaksh, 1984; Yaksh, 1979, 1984). Also, it has been reported that blockade of the spinal serotonergic or noradrenergic receptors by spinal injection of methysergide or yohimbine antagonize the antinociception induced by morphine administered supraspinally (Yaksh, 1979; Jensen and Yaksh, 1984; Wigdor and Wilcox, 1987). We observed in the present study that opioidergic and α_2-adrenergic receptors, but not serotonergic receptor, appear to be involved in orally administered eugenol-induced antinociception.

In conclusion, our results suggest that eugenol shows an antinociceptive property in various pain models related to inflammation, peripheral and central nerves pains. Furthermore, this antinociceptive effect of eugenol may be mediated by opioidergic and α_2-adrenergic receptors, but not serotonergic receptor.

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