



Original article

The possible mechanisms of protocatechuic acid-induced central analgesia

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ARTICLE INFO

Article history:

Received 22 November 2017

Accepted 1 February 2018

Available online 2 February 2018

Keywords:

Antinociception

Muscarinic receptors

 α 2-adrenoceptorsSerotonin 5-HT_{2A/2C} receptors

Opioidergic receptors

Protocatechuic acid

ABSTRACT

It is aimed to investigate the central antinociceptive effect of protocatechuic acid and the involvement of stimulation of opioidergic, serotonin 5-HT_{2A/2C}, α 2-adrenergic and muscarinic receptors in protocatechuic acid-induced central analgesia in mice. Time-dependent antinociceptive effects of protocatechuic acid at the oral doses of 75, 150 and 300 mg/kg were tested in hot-plate (integrated supraspinal response) and tail-immersion (spinal reflex) tests in mice. To investigate the mechanisms of action; the mice administered 300 mg/kg protocatechuic acid (p.o.) were pre-treated with non-specific opioid antagonist naloxone (5 mg/kg, i.p.), serotonin 5-HT_{2A/2C} receptor antagonist ketanserin (1 mg/kg, i.p.), α 2-adrenoceptor antagonist yohimbine (1 mg/kg, i.p.) and non-specific muscarinic antagonist atropine (5 mg/kg, i.p.), respectively. The antinociceptive effect of protocatechuic acid was observed at the doses of 75, 150 and 300 mg/kg in tail-immersion test, at the doses of 150 and 300 mg/kg in hot-plate test at different time interval. The enhancement in the latency of protocatechuic acid-induced response to thermal stimuli was antagonized by yohimbine, naloxone and atropine in tail-immersion test, while it was antagonized only by yohimbine and naloxone pretreatments in hot-plate test. These results indicated that protocatechuic acid has the central antinociceptive action that is probably organized by spinal mediated cholinergic and opioidergic, also spinal and supraspinal mediated noradrenergic modulation. However, further studies are required to understand how protocatechuic acid organizes the interactions of these modulatory systems. As a whole, these findings reinforce that protocatechuic acid is a potential agent that might be used for pain relief. Additionally, the clarification of the effect and mechanisms of action of protocatechuic acid will contribute to new therapeutic approaches and provide guidance for new drug development studies.

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1. Introduction

Phenolic compounds have gained attention since they are bioactive compounds. Protocatechuic acid (3,4-dihydroxybenzoic acid), an antioxidant phenolic acid, can reach tissues in amounts

which can exert biological effects on health (Semaming et al., 2015). It has been reported that protocatechuic acid shows antioxidant, antihyperglycemic and neuroprotective effects (Masella et al., 2012). Because Protocatechuic acid can easily crosses the blood brain barrier, it gains attention in the inhibition of neurodegenerative progress based on existing data (Krzysztoforska et al., 2017). Protocatechuic acid also possesses anti-inflammatory and antinociceptive effects in different animal models (Lende et al., 2011). More recently, Dhanshree et al., (2017) showed that the treatment with protocatechuic acid for 21 days increased the pain threshold in diabetic neuropathic pain model. Although the antinociceptive effect of protocatechuic acid was shown in a few studies (Lende et al., 2011), the mechanisms of antinociceptive action have not been clarified yet. Revealing the mechanism of action of drugs is highly important to identify the effect profiles of drugs and rational drug use. Pain perception and control process

Abbreviations: ATR, atropine; CTRL, control group; HT, serotonin; i.p., intraperitoneal; KTS, ketanserin; MPE, maximal possible effect; NLX, naloxone; p.o., per oral; PCA, protocatechuic acid; YOH, yohimbine; δ , delta; κ , kappa.

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Peer review under responsibility of King Saud University.



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from the periphery to the central nervous system are mediated through ascending and descending networks that include endogenous opioid-, monoamine-, and acetylcholine-mediated mechanisms via their own receptors (de Freitas et al., 2004; Kirkpatrick et al., 2015). This study aimed to investigate the time-dependent central antinociceptive effects of protocatechuic acid at the per oral doses of 75, 150 and 300 mg/kg, and the investigation of spinal and supraspinal organization of its antinociceptive effect by pre-treatment with non-specific opioid antagonist naloxone (5 mg/kg, i.p.), serotonin 5-HT_{2A/2C} receptor antagonist ketanserin (1 mg/kg, i.p.), α_2 -adrenoceptor antagonist yohimbine (1 mg/kg, i.p.) and non-specific muscarinic antagonist atropine (5 mg/kg, i.p.) before the administration of 300 mg/kg (p.o.) protocatechuic acid in hot-plate (integrated supraspinal response) and tail-immersion (spinal reflex) tests in mice.

2. Materials and methods

2.1. Animals

Adult Balb-c male mice was used in experimental studies. The mice were maintained at constant room temperature ($22 \pm 2^\circ\text{C}$) under a 12 h light–dark cycle with free access to standard food and water *ad libitum*. The animals received only water during the six hours preceding the experiments to avoid possible food interaction with protocatechuic acid. Analgesia tests were performed between 11:00 and 17:00 h. All experimental protocols were performed according to the principles and guidelines adopted by the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised in 1985) and approved by the Local Ethics Committee of Anadolu University and Osmangazi University, Eskisehir, Turkey.

2.2. Drugs and treatments

Protocatechuic acid ($\geq 97\%$ pure), α_2 -adrenoceptor antagonist yohimbine ($\geq 98\%$ pure), serotonin 5-HT_{2A/2C} receptor antagonist ketanserin ($>97\%$ pure), opioid antagonist naloxone ($\geq 98\%$ pure) and muscarinic receptor antagonist atropine ($\geq 99\%$ pure) (Sigma, St. Louis, MO, USA) were used in this study. All animals were randomly divided into groups of six animals each and baseline values of pain thresholds measured by hot-plate and tail-immersion tests. The first group was designated as control group that saline administered (p.o.) only (solvent vehicle). To the other three groups were orally treated with 75, 150 and 300 mg/kg protocatechuic acid by a gavage needle (18 G $\times$ 3 in. $\times$ 2.25 mm) which corresponds to an esophageal cannula. The antinociceptive effect of protocatechuic acid was tested time-dependently at 30, 45, 60, 90 and 120th minutes. Treatment schedule for mechanism of action studies as follows: pre-treatment with 5 mg/kg atropine 15 min before the 300 mg/kg protocatechuic acid administration, pre-treatment with 1 mg/kg ketanserin 30 min before the 300 mg/kg protocatechuic acid administration, pre-treatment with 1 mg/kg yohimbine 30 min before the 300 mg/kg protocatechuic acid administration, pre-treatment with 5 mg/kg naloxone 15 min before the 300 mg/kg protocatechuic acid administration, separately. All pre-treatments were performed as intraperitoneal (i.p.) route. The analgesia test procedures performed for mechanism of action studies were applied 45 min after 300 mg/kg protocatechuic acid administration since protocatechuic acid showed most significant antinociception at the dose of 300 mg/kg at 45 min.

2.3. Analgesia test procedures

2.3.1. Hot-plate test

The pain reflexes in response to a thermal stimulus were measured by using a Hot-Plate Analgesia Meter (No. 7280, Ugo Basile

Instruments, Comerio, Italy) (Eddy and Leimbach, 1953). The mice were gently put on the surface of the hot plate, set to $55 \pm 0.5^\circ\text{C}$. The latency of hind paw licking, hind paw flicking, or jumping was measured as reaction time. The cut-off time was taken as 20 s to minimize hind paw damage.

2.3.2. Tail-immersion test

The painful thermal stimuli was induced by dipping the tail tips of mice into a hot water bath (Heto, Allerød, Denmark) at $52.5 \pm 0.2^\circ\text{C}$ (Schmauss and Yaksh, 1984). The withdrawal latency of the tail from the hot water was noted as reaction time. The maximum cut-off time was 15 s to avoid the injury of tissues of the tail.

The results of the analgesia tests were expressed as a percentage of the maximal possible effect (MPE%) which was calculated over the latencies of response against thermal stimuli (Coelho et al., 2005):

$$\text{MPE\%} = \frac{[(\text{Postdrug latency}) - (\text{Predrug latency})]}{[(\text{Cutoff time}) - (\text{Predrug latency})]} \times 100$$

2.4. Data analyses

Statistical differences were analyzed using two-way ANOVA followed by Bonferroni method in antinociceptive action studies and one-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc tests in mechanism of action studies. The statistical analyses were carried out using GraphPad Prism version 5.0. The results were expressed as the mean $\pm$ standard error of the mean to show variation in groups. Differences were considered significant when $P \leq 0.05$.

3. Results

3.1. Central antinociceptive effect of protocatechuic acid

The MPE% values that describes the antinociceptive effect of protocatechuic acid in the hot-plate and tail-immersion tests are shown in Fig. 1 A and B, respectively. Protocatechuic acid at the doses of 150 and 300 mg/kg enhanced the response latency against thermal stimulus significantly ($P < 0.05$ and $P < 0.001$ for 150 mg/kg at 45 and 60th min, respectively; $P < 0.001$, $P < 0.001$ and $P < 0.01$ for 300 mg/kg at 45, 60 and 90th min, respectively) compared with the control group in the hot-plate test. 75, 150 and 300 mg/kg protocatechuic acid succeeded in increasing threshold significantly ($P < 0.01$ for 75 mg/kg at 60th min; $P < 0.05$, $P < 0.001$ and $P < 0.001$ for 150 mg/kg at 30, 45 and 60th min, respectively; $P < 0.001$ for 300 mg/kg at 30–60 min time interval) compared with the control group in the tail-immersion test.

3.2. Mechanism of action studies

Figs. 2 and 3 show that how pre-treatment of ketanserin (A), yohimbine (B), atropine (C) and naloxone (D) affect the MPE% values that describes the antinociceptive effect of 300 mg/kg protocatechuic acid in hot-plate and tail-immersion tests, respectively. The enhancement in the latency of 300 mg/kg protocatechuic acid-induced response to thermal stimuli was reversed significantly by yohimbine ($P < 0.01$) pre-treatment in hot-plate test, while it was reversed significantly by yohimbine ($P < 0.001$), naloxone ($P < 0.001$) and also atropine ($P < 0.01$) in tail-immersion test. The significant ($P < 0.001$) antinociception in 300 mg/kg protocatechuic acid-only group weakened in ketanserin and atropine pre-treated group ($P < 0.05$) in hot-plate test. The observed antinociception disappeared in naloxone pre-treated group although significant antagonism was not observed in hot-

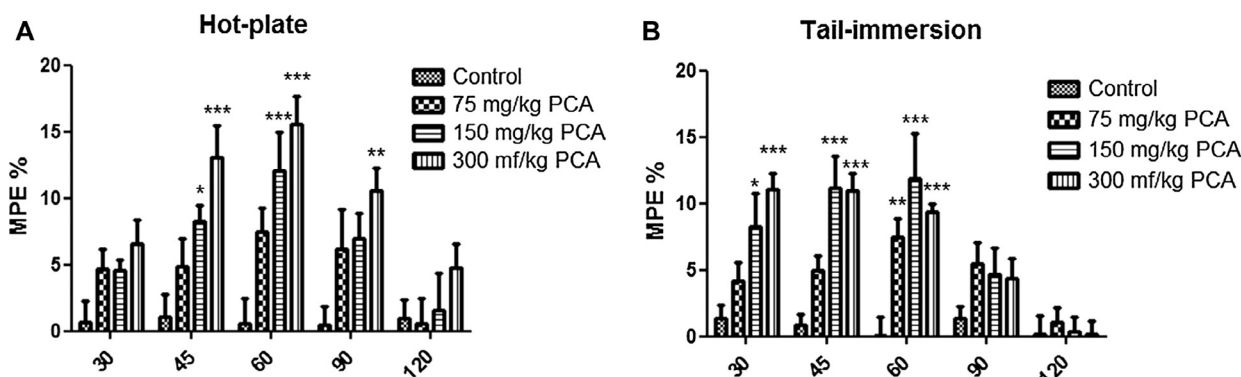


Fig. 1. The antinociception induced by 75, 150, and 300 mg/kg (po) protocatechuic acid in the hot-plate (A) and tail-immersion (B) tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$: significant difference from control. The statistical analyses were performed using two-way ANOVA, followed by Bonferroni multiple comparison tests. MPE%: The percentage of the maximal possible antinociceptive effect, PCA: Protocatechuic acid.

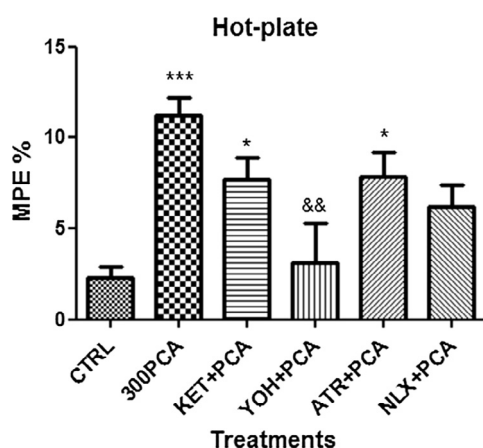


Fig. 2. The reversal effect of ketanserin (ip), yohimbine (ip), atropine (ip) and naloxone (ip) on 300 mg/kg (po) protocatechuic acid-induced antinociception in the hot-plate tests. * $P < 0.05$, *** $P < 0.001$: significant difference from CTRL. && $P < 0.01$: significant difference from 300 mg/kg PCA alone. The statistical analyses were performed using one-way ANOVA, followed by Newman-Keuls multiple comparison tests. MPE%: The percentage of the maximal possible antinociceptive effect, PCA: Protocatechuic acid, CTRL: Control group, KTS: Ketanserin, YOH: Yohimbine, ATR: Atropine, NLX: Naloxone.

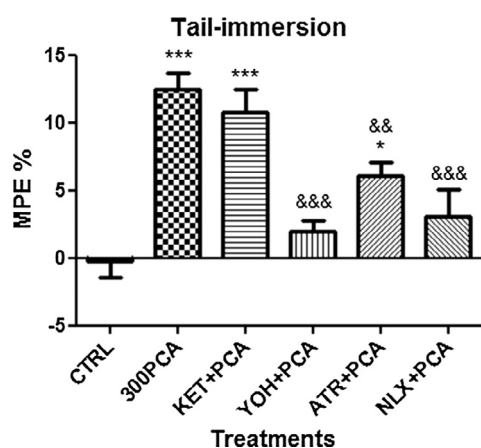


Fig. 3. The reversal effect of ketanserin (ip), yohimbine (ip), atropine (ip) and naloxone (ip) on 300 mg/kg (po) protocatechuic acid-induced antinociception in the tail-immersion tests. * $P < 0.05$, *** $P < 0.001$: significant difference from CTRL. && $P < 0.01$, &&& $P < 0.001$: significant difference from 300 mg/kg PCA alone. The statistical analyses were performed using one-way ANOVA, followed by Newman-Keuls multiple comparison tests. MPE%: The percentage of the maximal possible antinociceptive effect, PCA: Protocatechuic acid, CTRL: Control group, KTS: Ketanserin, YOH: Yohimbine, ATR: Atropine, NLX: Naloxone.

plate test. Additionally, the residual antinociceptive effect of 300 mg/kg protocatechuic acid in atropine pre-treated group in tail-immersion test were observed in spite of significant antagonism.

4. Discussion

The hot-plate and tail-immersion tests are used to assess pain response in basic pain research and testing the effectiveness of analgesics in small laboratory animals. It is possible to think that the chemicals which are enhanced the pain response in these methods are promising analgesics (Milind and Monu, 2013). It is concluded that protocatechuic acid shows antinociceptive effect since the enhancement of the latency of pain response against to thermal stimuli was observed. These methods also provide insight about the level of organization, spinal or supraspinal, of the effectiveness. The tail-immersion response is a simple spinal reflex; however, the hot-plate test is a behavioral model of nociception in which more organized behaviors such as hindpaw licking and jumping, reactions that are controlled by supraspinal mechanisms, are elicited (Flores et al., 2004). In pain control systems, the mechanism of supraspinal/spinal signal integration includes the release of a number of neurotransmitters, particularly endogenous opioids, noradrenaline, serotonin, and acetylcholine, and their activity (Fiorino and Garcia-Guzman, 2012). Thus, the involvement of these modulatory systems in protocatechuic acid-induced analgesia was investigated.

The presynaptic and postsynaptic α_2 -adrenoceptors mediate the antinociception through the noradrenaline stimuli. Noradrenergic pain control systems have dealt with spinal noradrenergic pain modulation and descending noradrenergic pain modulatory pathways that is originated from supraspinal region (Pertovaara, 2006). Yohimbine was used to antagonize the α_2 -adrenoceptors in this study. It antagonized the antinociception induced by protocatechuic acid in both the hot-plate and tail-immersion tests. Even the most pronounced antagonism was observed with yohimbine pre-treatment. Thereby it is thought that antinociceptive effect of protocatechuic acid may be primarily mediated by increased levels of noradrenaline and by activation of the spinal/supraspinal α_2 -adrenoceptors. However, the analgesia observed with protocatechuic acid may not only be modulated by noradrenergic system since the pain control is more complex process involved another interacting modulatory systems. Hence, alternative mechanisms of action need to be considered.

The opioidergic system plays a critical role in pain control by own self and it modulates other antinociceptive pathways as descending inhibitory pathways (Kanjhan, 1995). Nonselective

opioid antagonist naloxone was used for investigating the connection of opioidergic modulation with protocatechuic acid-induced antinociception, since almost all receptor subtypes, opioid μ (μ), kappa (κ), and delta (δ), mediate the antinociceptive effect of opioids (Al-Hasani and Bruchas, 2011). It was observed that spinal-organized opioidergic modulation is markedly involved in protocatechuic acid-induced analgesia similarly noradrenergic system. Additionally, it was also observed the apparent contribution of supraspinal-organized opioidergic modulation to antinociception of protocatechuic acid.

Serotonergic system has complex and important role in pain modulation especially through descending inhibitory pathways (Bardin, 2011). Although the various serotonin receptor subtypes are involved in the serotonin-induced antinociception, the role of serotonin 5HT_{2A/C} receptor-mediated response in protocatechuic acid-induced antinociception was investigated in this study with 5HT_{2A/C} receptor antagonist ketanserin. However, it was observed that there is no significant contribution of serotonergic system via 5HT_{2A/C} receptors. It is claimed that the analgesia induced by selective μ -opioid receptor agonists is closely related with the descending noradrenergic system but not related with the serotonergic system (Crisp et al., 1992; Ochi et al., 2002). It may be speculated that protocatechuic acid-induced antinociception is a selective μ -opioid receptor agonist-mediated analgesia. However, further detailed studies are needed to prove this speculation.

Additionally, the contribution of muscarinic cholinergic receptors in antinociception of protocatechuic acid was investigated by using atropine, since cholinergic receptors are also involved in inhibition response of pain (Duflo et al., 2005). It is thought that the cholinergic modulation of protocatechuic acid-induced analgesia is spinally mediated since atropine significantly antagonized the antinociceptive effect of protocatechuic acid only in the tail-immersion test.

It is well known that the stimulation of opioidergic receptors excites the spinal releasing of noradrenaline that causes directly stimulation of acetylcholine release in the spinal dorsal horn through the activation of the α_2 -adrenoceptors (Bouaziz et al., 1996; Chen and Pan, 2001; Obata et al., 2005; Pertovaara, 2006). Thus, it can be presumed that the antinociceptive effect of protocatechuic acid may be induced by stimulating the release of norepinephrine, especially as μ -agonists do, in the spinal cord, which acts on the excitatory α_2 -adrenoceptors on cholinergic neurons. However, this claim is needed to support by exhaustive biomolecular studies.

The partial antagonism observed in hot-plate test by pre-treatment with ketanserin, atropine and especially naloxone alongside dominant antagonism observed by pre-treatment with yohimbine should not be ignored. A5 (locus coeruleus), A6, and A7 (Kölliker-Füsse) nuclei are known as the main brain sites of the pain inhibitory effect of noradrenergic system. Noradrenergic projections to the spinal cord are originated from these noradrenergic nuclei (Pertovaara, 2006). Since the most significant reversing effect was provided by yohimbine pre-treatment in the hot-plate test, which assesses supraspinal pain behavior, it is thought that the mentioned central antinociceptive effect of protocatechuic acid is mainly controlled by the noradrenergic system. Thus, it is possible to conclude that protocatechuic acid may provide an effect that will activate this mentioned regions. Indeed, the partial role of other systems is not surprising since the central control of pain is not provided by a single system but by coordinated contribution of many neurotransmitters such as acetylcholine and endo-opioids. Further studies with combined antagonists may be performed to identify which systems and receptors are involved together in this effect.

In conclusion, protocatechuic acid has the central antinociceptive action in mice that is probably organized by spinal mediated

cholinergic and especially opioidergic and noradrenergic modulation. It is also thought that the activation of noradrenergic system via α_2 -adrenoceptors may mostly manage the antinociception of protocatechuic acid in supraspinal level. As a whole, these findings reinforce that protocatechuic acid is a potential agent that might be used for pain relief. Since protocatechuic acid is also safe with regard to good toxicity profile (Kakkar and Bais, 2014), this data make its potential more valuable. This investigation may provide a perspective to clinical trials to determine whether protocatechuic acid is effective in patients suffering from pain. Additionally, the clarification of the effect and mechanisms of actions of protocatechuic acid will contribute to new therapeutic approaches and provide guidance for new drug development studies.

5. Conflict of interest statement

The authors declare no conflicts of interest.

Acknowledgment

This study was supported by the Anadolu University Scientific Research Projects Unit (Project No: 1305S106), Anadolu University, Eskişehir, Turkey.

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