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5,6-, 8,9-, 11,12- and 14,15-Epoxyeicosatrienoic Acids (EETs) Induce Peripheral Receptor-Dependent Antinociception in PGE2-Induced Hyperalgesia in Mice

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Abstract

Epoxyeicosatrienoic acids (EETs) are cytochrome P450-epoxygenase-derived metabolites of arachidonic acid that act as endogenous signaling molecules in multiple biological systems, including their controversial effects on pain, including reports of the central analgesic effect and its action in inducing pain. The aim of this study was to verify the peripheral antinociceptive effect of EETs and the effect of the selective EET receptor antagonist, 14,15-EEZE, on this antinociception. The nociceptive threshold was determined by paw pressure withdrawal, and hyperalgesia was induced by intraplantar injection of PGE2 to evaluate the effect of EETs administration. EETs (5,6-, 8,9-, 11,12-, and 14,15-EET) were administered intraplantarly to male mice (n = 5). To examine the mechanism of action, a non-selective EET receptor antagonist (14,15-EEZE) was administered peripherally. Intraplantar injections of 5,6-, 8,9-, and 11,12-EET (32, 64, and 128 ng) or 14,15-EET (128, 256 and 512 ng), five minutes before the third hour after PGE2 injection induced a dose-dependent antinociceptive response. EETs showed peak action five minutes after injection, and this effect decreased concomitantly with a reduction in the nociceptive effect of PGE2 until approximately 100 min after injection (270 min after PGE2 injection). The maximum dose of each EET completely reversed the hyperalgesia induced by PGE2. The antinociceptive effect of EETs was confined to the paw that received the injection, indicating a localized effect. Intraplantar injection of the EET antagonist, 14,15-EEZE, reversed in a dose-dependent manner (32-512 ng/paw) the peripheral antinociception induced by 5,6-, 8,9-, 11,12-, and 14,15-EET. Our results provide evidence that EETs induce a peripheral antinociceptive effect and that the mechanism of action involves EET receptor activation.

Keywords: antinociception, EET, epoxyeicosatrienoic acids.

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Introduction

Epoxyeicosatrienoic acids (EETs) are metabolites of arachidonic acid produced by cytochrome P450 epoxygenases and are metabolized in the body through a variety of pathways, the most important being the soluble epoxide hydrolase pathway[1-2]. EETs are important signaling molecules that mediate and regulate a series of events, such as inflammation[3], cancer[4], angiogenesis[5] and cardiovascular diseases[6].

Among the various biological actions of EETs, the literature presents controversial results in the field of pain. Some studies have demonstrated that EETs are analgesics, which a reduction in lipopolysaccharide-induced thermal hyperalgesia was observed[7]. Terashvili et al. (2008) observed that 14,15-EET, but not other EETs, produced antinociception when injected into the periaqueductal gray matter, indicating a central analgesic effect of EETs[8]. In contrast, it was shown that 8,9-EET has a nociceptive effect in a zymosan-induced inflammatory model[9]. Furthermore, it has been shown that 5,6-EET causes central mechanical hypersensitivity[10]

Various bioactive lipid mediators regulate nociceptive pain and inflammation in peripheral tissues by interacting with receptor systems on primary sensory neurons and neighboring host-defense cells, such as macrophages, mast cells and keratinocytes[11].
EETs are important signaling molecules for mediating and regulating a range of local events, such as inflammation[3] and angiogenesis[5]. Soluble epoxide hydrolases inhibitors delivered through the transdermal route attenuated thermal hyperalgesia and mechanical allodynia in rats treated with LPS[7], indirectly indicating a peripheral effect for EETs. In light of these observation, this study aims to verify the peripheral effect of EETs on PGE2-hyperalgesia nociception and the involvement of their receptors, seeking to broaden the knowledge about these.

Methods

Animals

The experiments were performed on 30-40 g (10-12 weeks) male Swiss mice (CEBIO-ICB/UFMG, Belo Horizonte, Brazil). The calculated sample size was n=4/group, 216 in total. The animals were maintained in a temperature-controlled room on an automatic 12-hour light/dark cycle, with free access to food and water. All tests were carried out during the light phase and animals were randomly selected. The animal experimental protocols were approved by the UFMG Ethics Committee on the Use of Animals (protocol 75/2017) and all animal care are in accordance with the recommendations for the evaluation of experimental pain in animals[12].

Measurement of Nociceptive Threshold

Hyperalgesia was induced by subcutaneous injection of PGE2 (2 µg/paw) into the mice's plantar surface hind paw. The mechanical nociceptive threshold was assessed by measuring the response to a paw pressure test, and adapted to mice[13]. An algesimeter (Ugo-Basile, Italy), which consisted of a cone-shaped paw-presser with a rounded tip, was used to apply linearly increasing pressure to the hind paw. The weight in grams required to elicit a nociceptive response to paw withdrawal was defined as the nociceptive threshold. A cut-off value of 160 g was used to prevent possible damage to the paws. The nociceptive threshold was measured in the hind paw and determined as the average of three consecutive trials recorded before (baseline nociceptive threshold) and at different time points after the PGE2 and EETs injections. The results are expressed in grams. To minimize stress, the mice were habituated to the apparatus two days prior to the experiments.

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Drugs

5,6-, 8,9-, 11,12-, and 14-15 epoxyeicosatrienoic acid (Cayman Chemical, USA) were diluted in ethanol 6.4% in saline. 14,15-Epoxyeicosa-5(Z)-enoic acid (Cayman Chemical, USA) was diluted in ethanol 6.4% in saline. Prostaglandin E2 (Sigma, USA), the hyperalgesic agent, was diluted in ethanol 10% in saline. All the drugs were subcutaneously injected into the plantar surface of the right paw in a volume of 20 µL per paw.

Experimental Protocol

In all experiments, the baseline nociceptive threshold of each animal was measured before the injection of any substance. To evaluate the temporal development of the dose-response curve of each EET, these drugs were injected 5 min prior to the peak of action of PGE2-induced hyperalgesia (180 min). Nociceptive threshold measurements were performed at different time points from 180 to 300 min after the first injection. To determine whether each EET acted only peripherally, PGE2 was injected into both hind paws. The highest dose of each EET was administered only to the right paw, while the contralateral paw received the vehicle (ethanol in saline). Nociceptive threshold measurements were taken in both hind paws to exclude systemic effects. For these experiments and the EETs antagonist protocol, the nociceptive threshold was measured before any injection (zero time) and 180 min after PGE2 injection (peak action). The difference between these values was expressed as the Δ of the nociceptive threshold. The protocols follow previous experiments and studies already published by our research group[14].

Statistical Analysis

Results are presented as the mean ± standard error of the mean (S.E.M.). Statistical analysis was carried out using Graph Prism 8.0.2 software and the data were analyzed by analysis of variance (ANOVA) followed by Bonferroni test. Statistically significance was set at $p<0.05$.

Results and Discussion

Antinociception of EETs on PGE2-induced Nociception in the Paw of Mice and Exclusion of the Systemic Effect

Injection of 2 µg PGE2 into the plantar hind paw skin caused nociceptive threshold (NT) to decrease significantly at all timepoints across a 4-h period (Figs 2,3,4 and 5, solid red circle), which had a peak effect at three hours where NT decreased to maximal of baseline. Vehicle-treated control (black solid circle) showed no significant changes in NT over the same 4-h testing period, comparing to basal measurement.

To evaluate the potential peripheral antinociceptive effects induced by different EETs, dose-response curves were used over time against hyperalgesia induced by PGE2. Intraplantar injection of 5,6-, 8,9-, and 11,12-EET (32, 64, and 128 ng) or 14,15-EET (128, 256, and 512 ng), five minutes before the third hour after PGE2 injection (peak action of this substance), induced a dose-dependent antinociceptive response (Figs 2,3,4 and 5). All evaluated EETs showed a similar response profile, although 14,15-EET, despite having the same efficacy, was less potent than the others EETs. EETs showed peak action five minutes after injection, and this effect decreased, concomitantly with a reduction in the nociceptive effect of PGE2 until approximately 100 min after injection (270 min after PGE2 injection). The maximum dose of each EET completely reversed the hyperalgesia induced by PGE2. We tested for potential analgesic effects of EETs in the absence of inflammatory pain, and the same maximal doses of them did not significantly change NT (not shown).

Among the different substances that can induce hyperalgesia, in this study, PGE2 was used, whose nociceptive effect is related to the ability of this substance to decrease the activation threshold of nociceptive primary afferent neurons due to an increase in neuronal excitability[15]

The pronociceptive PGE2 effect is thought to be caused by activation of Gs protein-coupled EP2 and EP4 receptors in nociceptive neurons and involves cAMP synthesis[16]. Mechanistically, cAMP-dependent pathways phosphorylate neuronal voltage-gated sodium channels (NaV) necessary for action potential generation[17]. Sensitization of nociceptors induced by PGE2 does not depend on the participation of cells or intermediate mediators, and nociceptors are directly activated in vitro by high concentrations of PGE2[18]. Therefore, using a pain model induced by PGE2 eliminates the possibility that the effect of the studied substance is the result of blocking in the release or action of mediators involved in the inflammatory process[19].

To exclude possible systemic effects, PGE2 was administered at time zero in both hind paws, and each EET at its highest dose was injected only in the right hind paw five minutes before the third hour after PGE2 injection. Measurements of the nociceptive threshold of both hind paws were taken immediately before and three hours after the intraplantar injection of PGE2, and the difference between the means of the measurements was calculated (Δ of the nociceptive threshold). At their highest doses, 5,6-, 8,9-, 11,12- and 14,15-EET, induced an effect restricted to the treated paw without changing the PGE2-induced hyperalgesia in the contralateral paw, indicating that these doses of EET are only locally effective (Insert Figs 2, 3, 4, 5).

In the present study, the hyperalgesic agent PGE2 was used as an experimental model to verify whether EETs have peripheral antinociceptive effects. Our results demonstrated that 5,6-, 8,9-, 11,12- or 14,15-EET, when injected into the mouse paw, induced peripheral antinociception against PGE2 hyperalgesia which was restricted to the treated paw and did not alter the hyperalgesia induced by PGE2 in the contralateral paw, indicating that the maximum doses of EET used were only effective locally. These data are in agreement with those of previous studies showing that topical administration of a mixture of EETs reduced thermal hyperalgesia in a pain model induced by the injection of lipopolysaccharide (LPS) in the rats' paw[7]. Furthermore, it has been shown that intracerebroventricular injection of 14,15-EET also induces antinociception[8]. Similarly, it was demonstrated that intraplantar injection of 5,6-EET induces mechanical, but not thermal, nociception, and this event is dependent on transient receptor potential ankyrin 1 (TRPA1).10 In contrast, 8,9-EET induces mechanical, but not thermal, hyperalgesia[9].

Effect of 14,15-EEZE on EETs-induced Antinociception

Intraplantar injection of the EET antagonist, 14,15-EEZE, reversed in a dose-dependent manner the peripheral antinociception induced by 5,6-, 8,9-, 11,12- and 14,15-EET (Fig. 6). When administered alone using the same protocol, the highest dose of the antagonist did not change the response to PGE2 or the vehicle (Fig. 6E).

EETs are responsible for diverse biochemical and functional responses; therefore, it is believed that more than one mechanism or signal transduction pathway is responsible for all their actions. Some functional effects of EETs, such as their ability to regulate gene

expression, suggest an intracellular action of these substances[20,21]. However, other studies indicate that EETs act via membrane-binding sites or receptors.22-24 Studies suggest that EET actions are partly, mediated by signaling from G protein-coupled receptors (GPCRs). Therefore, possible candidate GPCRs for EET receptors were selected, and it was observed that EETs, as well as other free fatty acids, bind with low affinity to GPR40 and GPR132 receptors in hematopoietic and vascular endothelium cells[25,26]. The authors report that these GPCRs are related with increase of intracellular calcium concentration and also with ERK phosphorylation. However, despite an intense search for the molecular mechanisms underlying the biological actions of EETs, these are still not fully understood, and the identity of EET receptor remains unknown. Later, binding study provided a potential mechanism of action for this analgesia by determining that EETs bind the peripheral benzodiazepine receptor also known as the translocator protein (TSPO)[27].

The literature has already identified the structural requirements for the biological activity induced by EETs, making it possible to characterize specific antagonists of these substances[28]. Among the non-selective pharmacological antagonists of EETs, 14,15-EEZE, has been shown to inhibit vascular relaxation induced by 5,6-, 8,9-, 11,12- and 14,15-EET in bovine coronary arteries[29]. Furthermore, the same antagonist reduced the protective action of exogenous and endogenous EETs in dog hearts, and inhibited cell motility in prostate carcinoma induced by 11,12-EET[30].

Antagonists are important pharmacological tools for identifying the biological actions of substances that are objects of study. Therefore, we used the EETs antagonist 14,15-EEZE to demonstrate the peripheral antinociceptive effects of these substances on PGE2-induced hyperalgesia. This antagonist reversed the peripheral antinociceptive effects of EETs, confirming that these substances induce antinociception via receptor activation.

Conclusion

Our results provide evidence that EETs induce a peripheral antinociceptive effect in PGE2-induced pain model. Studies found in the literature have reported isolated effects of EETs related to central analgesia8 and even pain9,10, in addition to indirect evidence for the inhibition of their synthesis7. While these assays in mice have limitations in their predictive capacities, they do show a robust and reproducible antihyperalgesic response, opening up possibilities for investigating this class of substance with perspectives for its therapeutic application. The use of the AX antagonist suggests the participation of specific receptors in the peripheral antinociceptive action of the EETs evaluated.

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Conflict of Interest

None declared.

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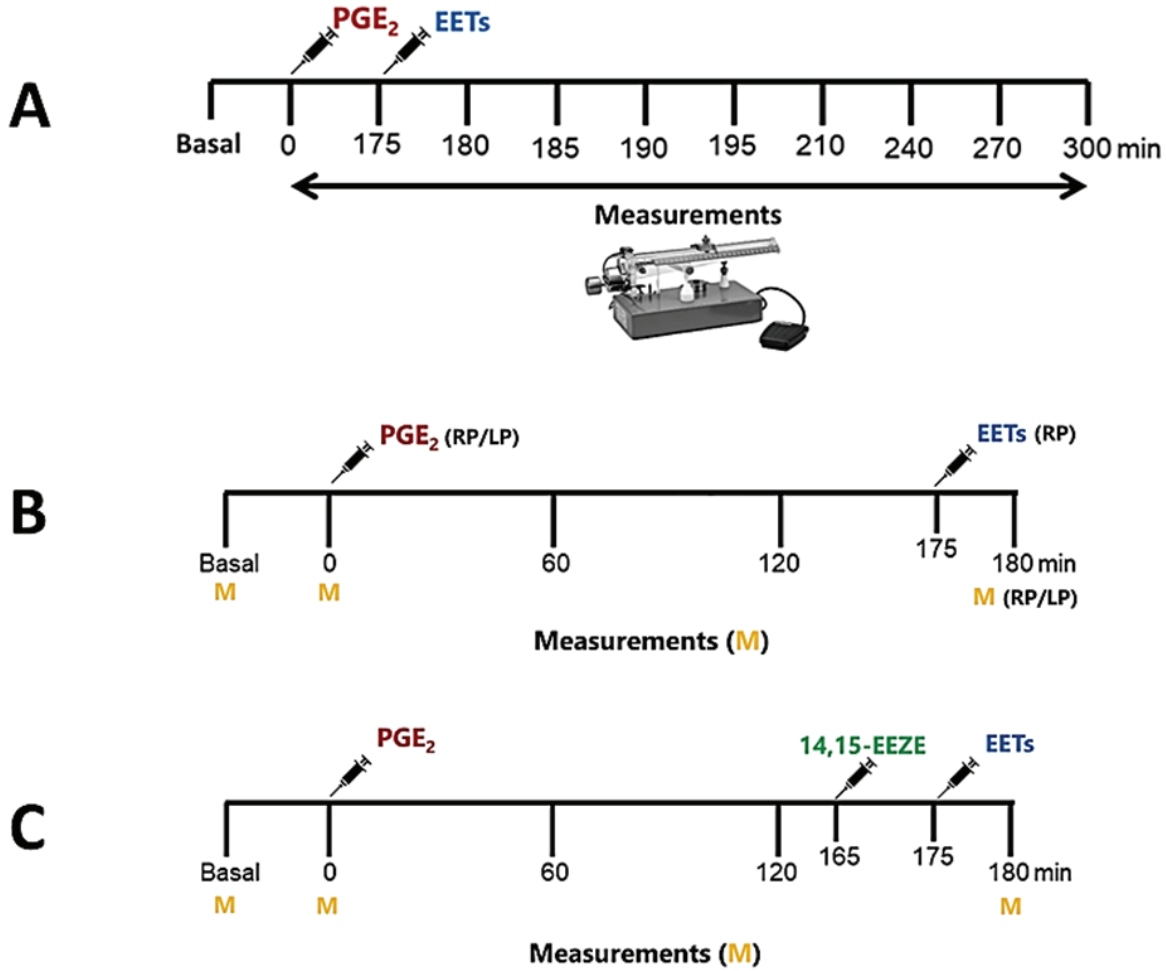

Figure 1. (A) Experimental protocol for the temporal evaluation of the antinociceptive effect of EETs. (B) Exclusion of the Systemic antinociceptive effect of EETs. PGE2 was administered at 0 min in the right (RP) and left (LP) hind legs of the animals, while EETs, at their maximum doses, were administered after 175 min only in the RP and its vehicle in the LP. Measurements of the nociceptive threshold of both paws were made before and after 180 min of PGE2 injection (time at which the maximum effect is observed). (C) Effect of EET receptor antagonist (14,15-EEZE) on EETs-induced Antinociception. In the experiments evaluating EETs receptors involved in antinociception induced by EETs, the Δ of the nociceptive threshold was used, which refers to the difference between the nociceptive threshold obtained at the beginning of the experiment before any injection (baseline value) and the threshold measured after 180 min PGE2, Prostaglandin E2; EETs, Epoxyeicosatrienoic acids.

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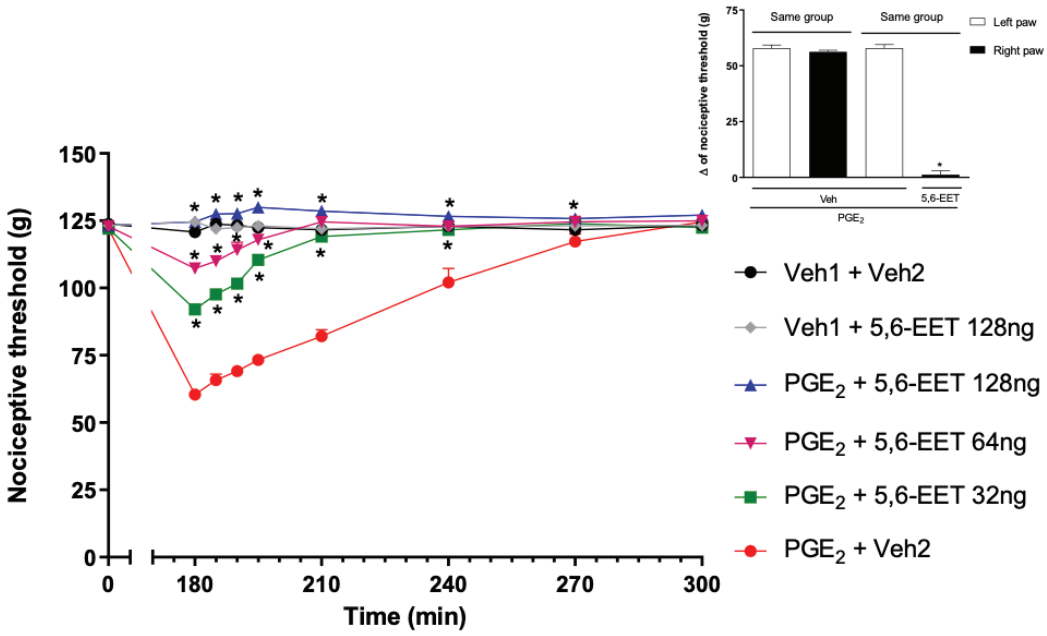

Figure 2. Antinociceptive effect of intraplantar injection of 5,6-EET in PGE2-induced hyperalgesia. The graph shows the time course of the peripheral antinociceptive effect of different doses of 5,6-EET, and the insert shows the exclusion of systemic antinociceptive effect of 5,6-EET (128 ng) in hyperalgesic paws; $F(3, 12) = 383.9$. Data are presented as mean ± SEM (n = 4). * indicates a significant difference compared with PGE2 + Veh ($P<0.05$), ANOVA with Bonferroni post-test. Veh= Vehicle. Veh1 = ethanol 10%, Veh2 = methyl acetate 6.4%. $F(5, 18) = 253.2$. PGE2= Prostaglandin E2; EETs= Epoxyeicosatrienoic acids.

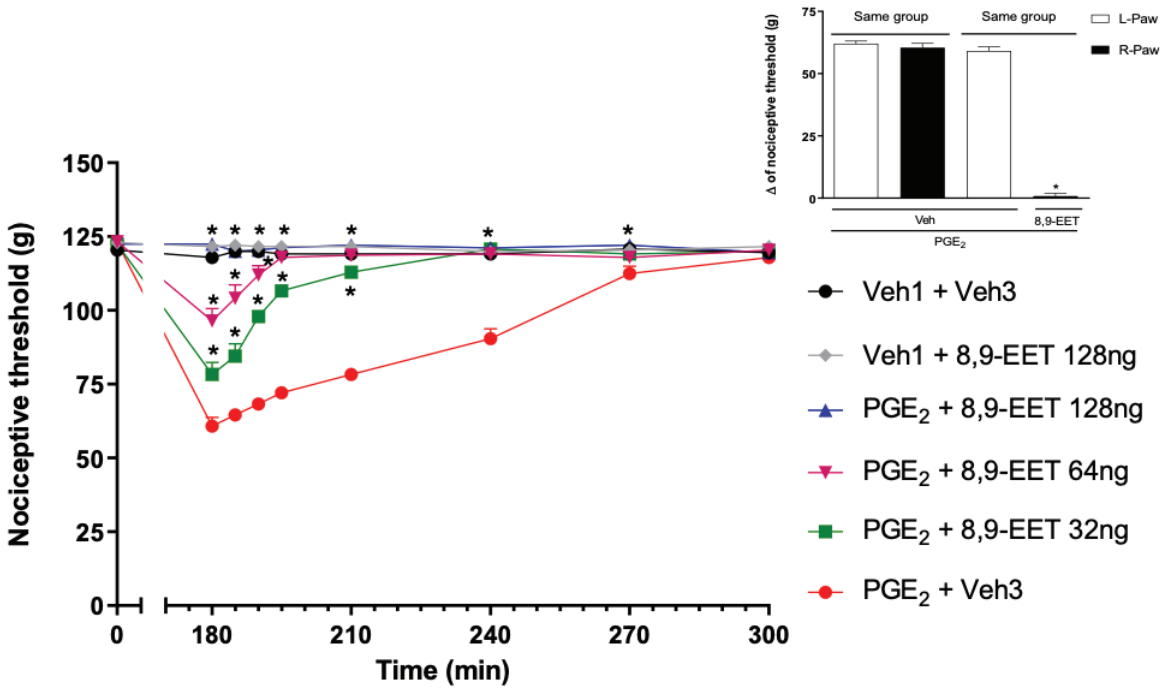

Figure 3. Antinociceptive effect of intraplantar injection of 8,9-EET in PGE2-induced hyperalgesia. The graph shows the time course of the peripheral antinociceptive effect of different doses of 8,9-EET, and the insert shows the exclusion of systemic antinociceptive effect of 8,9-EET (128 ng) in hyperalgesic paws; $F(3, 12) = 462.4$. Data are presented as mean ± SEM (n = 4). * indicates a significant difference compared with PGE2 + Veh ($P<0.05$), ANOVA with Bonferroni post-test. Veh1 = ethanol 10%, Veh2 = ethanol 6.4%. $F(5, 18) = 117.6$.

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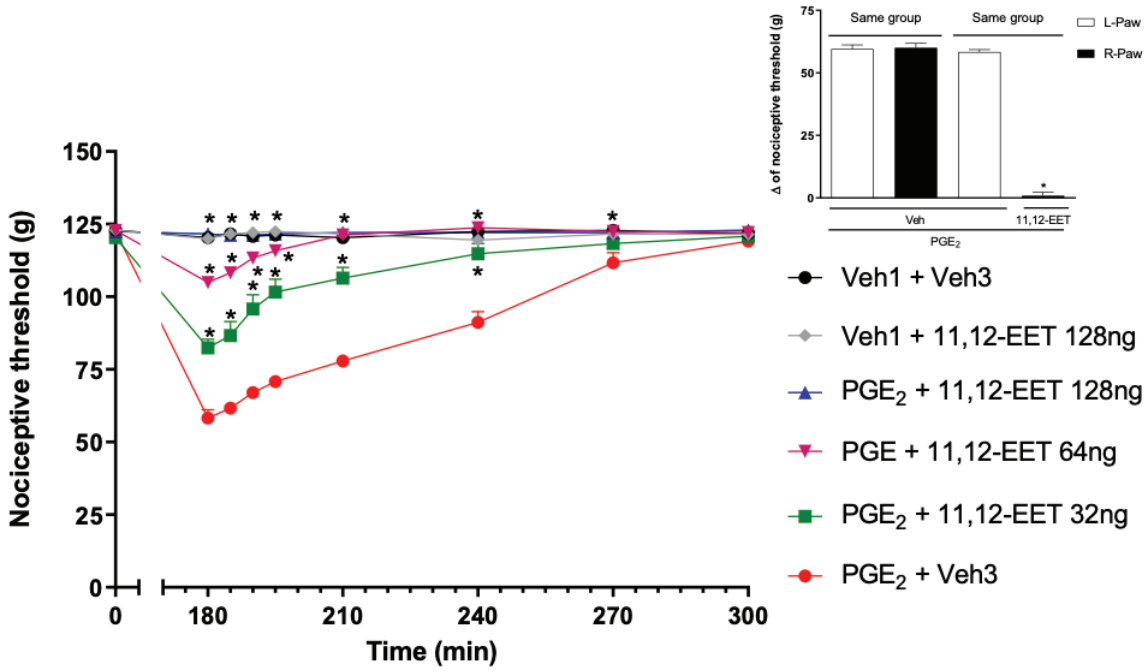

Figure 4. Antinociceptive effect of intraplantar injection of 11,12-EET in PGE2-induced hyperalgesia. The graph shows the time course of the peripheral antinociceptive effect of different doses of 11,12-EET, and the insert shows the exclusion of systemic antinociceptive effect of 11,12-EET (128 ng) in hyperalgesic paws; F(3, 12) = 395.7. Data are presented as mean ± SEM (n = 4). * indicates a significant difference compared with PGE2 + Veh ($P<0.05$), ANOVA with Bonferroni post-test. Veh1 = ethanol 10%, Veh2 = ethanol 6.4%. F(5, 18) = 113.2.

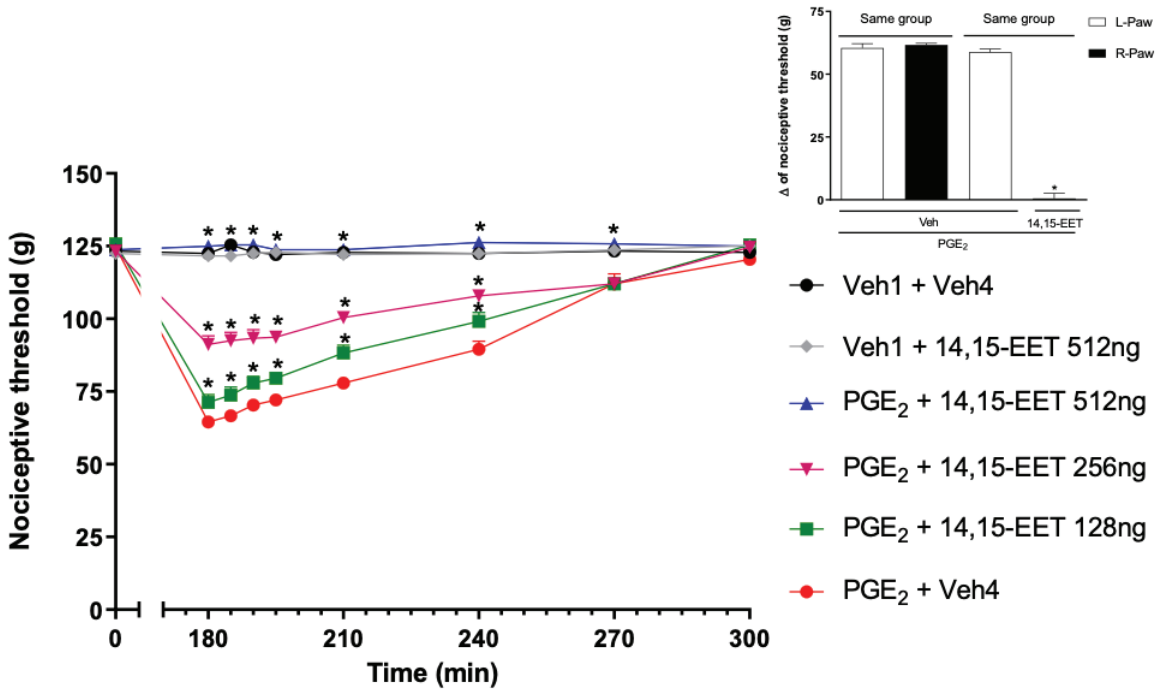

Figure 5. Antinociceptive effect of intraplantar injection of 14,15-EET in PGE2-induced hyperalgesia. The graph shows the time course of the peripheral antinociceptive effect of different doses of 14,15-EET, and the insert shows the exclusion of systemic antinociceptive effect of 14,15-EET (512 ng) in hyperalgesic paws; $F(3, 12) = 392.3$. Data are presented as mean ± SEM (n = 4). * indicates a significant difference compared with PGE2 + Veh ($P<0.05$), ANOVA with Bonferroni post-test. Veh1 = ethanol 10%, Veh2 = ethanol 25.6%. $F(5, 18) = 178.4$.

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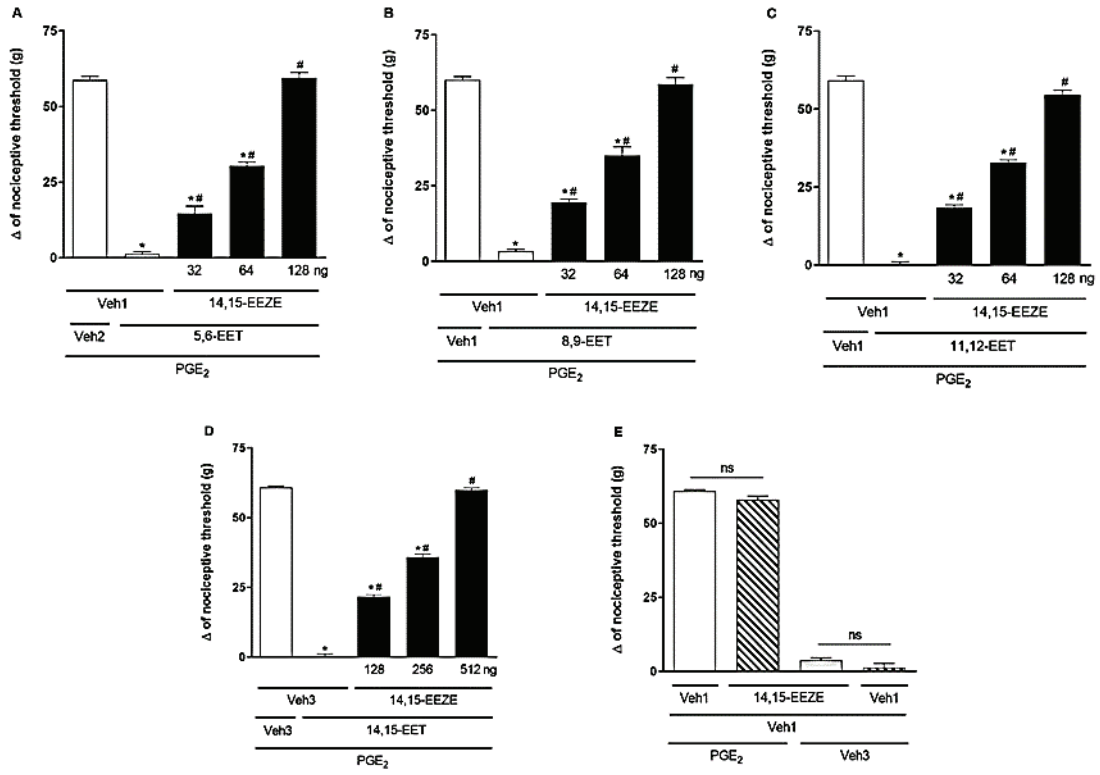

Figure 6. Effect of the pretreatment with EETs receptor antagonist on the EETs-induced peripheral antinociception against the hyperalgesia induced by PGE2. 14,15 EEZE (32, 64, 128, 256 and 512 ng/paw) was injected into the right hind paw 10 min prior to the intraplantar injection of (A) 5,6-, (B) 8,9-, (C) 11,12-, (D) 14,15-EET (128, 128, 128 and 512 ng/paw, respectively) and (E) Vehicle. EETs were given 175 min after the local injection of prostaglandin E2 (PGE2; 2 µg/paw). Measurements were made at 180 min. Each column represents the mean ± SEM (n=4). Veh1 = ethanol 6.4%, Veh2 = methyl acetate 6.4%, and Veh3 = ethanol 10%. * $p<0.05$ compared to the PGE2 + Veh + Veh and # $p<0.05$ compared to the PGE2 + Veh1 + EET; one-way ANOVA followed by the Bonferroni test. (A) $F(4, 15) = 275.7$; (B) $F(4, 15) = 189.1$; (C) $F(4, 15) = 455.3$, (D) $F(4, 15) = 997.4$, (E) $F(3, 12) = 965.5$.