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Role of Endothelial Cells in Antihyperalgesia Induced by a Triptan and β-blocker

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Abstract

While blood vessels have long been implicated in diverse pain syndromes (e.g., migraine headache, angina pectoris, vasculitis, and Raynaud's syndrome), underlying mechanisms remain to be elucidated. Recent evidence supports a contribution of the vascular endothelium in endothelin-1 induced hyperalgesia, and its enhancement by repeated mechanical stimulation; a phenomenon referred to as stimulus-induced enhancement of (endothelin) hyperalgesia (SIEH). SIEH is thought to be mediated by release of ATP from endothelial cells, to act on P2X3 receptors on nociceptors. In the present study we evaluated the ability of another vasoactive hyperalgesic agent, epinephrine, to induce endothelial cell dependent hyperalgesia and SIEH. We found that epinephrine also produces hyperalgesia and SIEH. Both a P2X3 receptor antagonist, A317491 and octoxynol-9, which attenuate endothelial cell function, eliminated SIEH without affecting epinephrine hyperalgesia. We further evaluated the hypothesis that members of two important classes of drugs used to treat migraine headache, whose receptors are present in endothelial cells - the triptans and beta blockers - have a vascular component to their anti-hyperalgesic action. For this, we tested the effect of ICI-118,551, a β_2-adrenergic receptor antagonist and sumatriptan, an agonist at 5-HT_{1B} and 5-HT_{1D} receptors, on nociceptive effects of endothelin and epinephrine. ICI-118,551 inhibited endothelin SIEH, and attenuated epinephrine hyperalgesia and SIEH. Sumatriptan inhibited epinephrine SIEH and inhibited endothelin hyperalgesia and SIEH, while having no effect on epinephrine hyperalgesia or the hyperalgesia induced by a prototypical direct-acting inflammatory mediator, prostaglandin E_2. These results support the suggestion that triptans and beta-blockers interact with the endothelial cell component of the blood vessel to produce anti-hyperalgesia.

Keywords

Endothelin; Epinephrine; Hyperalgesia; Triptan; β-blocker; Endothelial cell

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INTRODUCTION

Clinical evidence supports a role of endothelial mechanisms in vascular pain. Genetic studies in patients, have described an association between migraine and both ET-AR and ET-BR endothelin receptors (Tzourio et al., 2001, Lemos et al., 2011) and elevated plasma levels of endothelin have been found during acute migraine attacks (Farkkila et al., 1992, Kallela et al., 1998). β -blockers and triptans (Diener et al., 2008, Chiam, 2012), two important and effective classes of drugs used to treat migraine headache have their targets, β-adrenergic and $5HT1_B$ and $5HT1_D$ receptors (Diener and Limmroth, 2005, Evans et al., 2008), on endothelial cells (McLeod and Piper, 1992a) as well as on nociceptors (Potrebic et al., 2003).

We have recently shown that mechanical hyperalgesia induced by the potent vasoconstrictor, endothelin-1, is enhanced by repeated mechanical stimulation (Joseph et al., 2011). This stimulus-induced enhancement of endothelin hyperalgesia is not produced by action of endothelin-1 on the nociceptor, as antisense to mRNA for the ET-AR endothelial receptor, administered intrathecally so that nociceptors are the only cell at the site of nociceptive testing exposed to it, inhibited endothelin-1 hyperalgesia but not its enhancement by mechanical stimuli (Joseph E.K. et al., 2012, in review). Conversely, injury to endothelial cells, which line the blood vessel lumen, eliminated stimulus-induced enhancement of endothelin-1 hyperalgesia, without attenuating its hyperalgesia (Joseph E.K. et al., 2012, in review).

In the present experiments, we have tested the hypothesis that the hyperalgesia induced by β_2-adrenergic receptor agonists, which are vasoactive, is also enhanced by mechanical stimulation and that this enhancement is also endothelial cell dependent. We also tested the hypothesis that representatives of two major classes of anti-migraine drugs, triptans and beta-blockers, are capable of inhibiting stimulus-induced enhancement of the hyperalgesia produced by endothelin-1 and the β-adrenergic agonist epinephrine, and that endothelial cells play a role in these actions.

METHODS

Animals

Experiments were performed on adult male Sprague Dawley rats (200–250 g; Charles River, Hollister, CA). Animals were housed three per cage, under a 12-h light/dark cycle, in a temperature and humidity controlled environment. Food and water were available *ad libitum*. All behavioral nociceptive testing was performed between 10:00 am and 4:00 pm. Rats were acclimatized to the experimental area and behavioral procedures prior to the experiment. To acclimatize rats to the testing environment, they were brought to the experimental area in their home cages and left in the cages for 15–30 min after which they were placed in a restrainer (cylindrical transparent acrylic tubes that have openings on their sides, to allow extension of the hind legs from the restrainer, for nociceptive testing). Rats were left in the restrainer for another 15–30 min before nociceptive testing was started. This acclimatization procedure consistently results in baseline paw withdrawal thresholds of 100 – 120 g for the body weight range for the rats used in this study. All experimental protocols

were approved by the UCSF Committee on Animal Research and conformed to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

Nociceptive testing

The nociceptive flexion reflex was quantified with an Ugo Basile Analgesymeter® (Stoelting, Chicago, IL), which applies a linearly increasing mechanical force to the dorsum of the rat's hind paw. Nociceptive threshold was defined as the force, in grams, at which the rat withdrew its hind paw. Baseline nociceptive thresholds, was defined as the mean of three readings taken at 5-min intervals, determined prior to all experiments. The mean of these three readings was considered to be the baseline paw-withdrawal threshold before drug administration. Mechanical threshold was re-determined at four time points (15, 20, 25 and 30 min) after treatments and this value was used to calculate the percentage change from the baseline threshold for each paw. Each paw was treated as an independent measure; both paws of the same rat received the same treatment (Aley and Levine, 1997, Khasar et al., 1998, Aley and Levine, 1999, Joseph and Levine, 2006). Each experiment was performed on separate groups of rats. Each animal acted as its own control, with inhibitor injected intradermally into both hind paws 15 min prior to the administration of endothelin-1 or epinephrine and paw withdrawal thresholds compared, pre- and post-drug treatment. Hyperalgesia was defined as a decrease in mechanical nociceptive threshold, here presented as percent reduction from baseline [% reduction in threshold = (pretreatment threshold − post-treatment threshold)/ (pretreatment threshold)] × 100.

Stimulus-induced enhancement of hyperalgesia protocol

In general, the mechanical thresholds determined at four time points (15, 20, 25 and 30 min) after treatments are similar. However, in endothelin and epinephrine treated rats, there appeared further enhancement of hyperalgesia with repeated testing of mechanical nociceptive threshold (i.e., further decrease in paw withdrawal threshold with each repeated application of the stimulus), which we refer to as stimulus-induced enhancement of hyperalgesia. To differentiate the stimulus-induced enhancement of hyperalgesia from the mechanical hyperalgesia (1^{st}, 2^{nd}, 3^{rd} and 4^{th} reading, taken at 5 min intervals (15–30 min)) induced by both endothelin-1 and epinephrine, the paw withdrawal thresholds were measured (single reading) at an early (5 min) and late (30 min) time point, in different groups of rats, and these results are also shown in the relevant figure.

Drugs

The drugs employed in this study were: endothelin-1, epinephrine, ICI-118,551 (β_2-adrenergic receptor antagonist), sumatriptan ($5HT_{1B}$ and $5HT_{1D}$ agonist), A-317491, octoxynol-9, and prostaglandin E_2 (all from Sigma-Aldrich, St. Louis, MO). Drug doses employed in this study were based on dose response curves generated in our previous studies (Aley and Levine, 1999, Khasar et al., 1999, Joseph et al., 2011) or the dose response curves performed as part of the present study.

All drugs except octoxynol-9 were administered intradermally (i.d.) in a volume of 5 μl using a 30-gauge hypodermic needle attached to a micro-syringe (Hamilton, Reno, NV) by

PE-10 polyethylene tubing. Octoxynol-9 (0.5% V/V, 1 ml/kg weight) was administered intravenously through a tail vein. All inhibitors were administered 15 min prior to endothelin-1, epinephrine or prostaglandin E_2 (PGE_2) and nociceptive thresholds measured (four times), at 15, 20, 25 and 30 min post endothelin-1, epinephrine or prostaglandin E_2 administration. Also, paw withdrawal thresholds were determined by a single reading at 5' and 30' post endothelin and epinephrine administration, to differentiate the time course of mechanical hyperalgesia from the enhancement of hyperalgesia induced by repeated stimulation. The *per se* effect of all the inhibitors including octoxynol-9 were separately evaluated and none had significant effect on the basal paw-withdrawal threshold of the naïve rats (data not shown). All drugs were dissolved in saline.

Endothelial cell injury

In the cardiovascular and renal literature, a role of endothelial cells in vascular function has been evaluated, *in vivo* and *in situ*, using brief exposure to octoxynol-9. As shown by light and electron microscopy, the intravenous or intra-arterial administration of octoxynol-9 selectively injures the blood vessel endothelial cell (McLeod and Piper, 1992b, Bourreau et al., 1993). Octoxynol-9 is used both *in vivo* (Pridgen et al., 2011, Enanche and Volanschi, 2012) and *in vitro* (Eddy et al., 2012, Murphy et al., 2012, Soni et al., 2012). While new to the field of pain research, octoxynol-9 has been used for experimental intervention in cardiovascular studies for many years (Connor et al., 1989, Sarvazyan, 1998, Mink et al., 2007).

To evaluate the role of the endothelial cell in stimulus-induced enhancement of hyperalgesia, rats received an intravenous injection, through a tail vein, of a 0.5% solution of octoxynol-9, at a volume of 1 ml/kg body weight (Joseph EK et al., 2012, in review). ET-1 was injected 15 minutes later, and the animals evaluated for hyperalgesia and stimulus-induced enhancement of this hyperalgesia. Injection of saline (diluent for octoxynol-9) served as the control. Rats showed no indication of distress throughout the period of the experiment following administration of octoxynol-9.

Statistical analysis

In all experiments, the dependent variable was change in paw withdrawal threshold, represented as percentage change from the pre-treatment baseline threshold or from the corresponding controls. Group data are represented as mean ± SEM. Statistical significance was determined by two-way repeated measures ANOVA followed by Bonferroni post hoc test comparing results within and between groups at different time points. P values <0.05 were considered statistically significant.

RESULTS

Beta$_2$-adrenergic agonist

We have previously demonstrated that while endothelin-1 induces mechanical hyperalgesia that is enhanced by the mechanical stimulus used to test nociceptive threshold (Joseph et al., 2011), multiple other hyperalgesia-inducing mediators (i.e., prostaglandin E_2 (PGE_2), nerve growth factor (NGF), glia-derived neurotrophic factor (GDNF), interleukin-6 (IL-6) and

tumor necrosis factor alpha (TNFα)) are not associated with stimulus-induced enhancement of their hyperalgesia (Joseph et al., 2011). β-adrenergic agonists produce mechanical hyperalgesia (Khasar et al., 1999), and since β-adrenergic receptors are present on endothelial cells (Queen et al., 2006, Grueb et al., 2008), we tested the hypothesis that mechanical stimulation also enhances hyperalgesia induced by epinephrine, an endogenous ligand for β-adrenergic receptors.

The intradermal injection of epinephrine (100 ng) produced mechanical hyperalgesia and stimulus-induced enhancement of epinephrine hyperalgesia. A single reading of the paw withdrawal threshold at 5 min or 30 min following the epinephrine administration produced a similar degree of hyperalgesia, whereas repeated readings (indicated by 1^{st}, 2^{nd}, 3^{rd} and 4^{th} taken at 5 min intervals, over a similar time period after injection of epinephrine) produce a significant increase from that of a single reading (Fig. 1). To confirm that this stimulus-induced enhancement of epinephrine hyperalgesia was, like that induced by endothelin-1, endothelial cell mediated, a group of rats were pretreated with octoxynol-9, which eliminates the stimulus-induced enhancement of endothelin-1 hyperalgesia (Joseph E.K. et al., 2012, in review). In this group of rats, while epinephrine-induced hyperalgesia was unaffected, epinephrine failed to produce stimulus-induced enhancement of epinephrine hyperalgesia (Fig. 1, n = 8/group).

To further confirm that this stimulus-induced enhancement of epinephrine hyperalgesia was, like that induced by endothelin-1, P2X3 or P2X2/3 receptor dependent, we tested the effect of A-317491, a P2X2/3 inhibitor on stimulus-induced enhancement of epinephrine hyperalgesia. A group of rats were treated with A-317491 (1 μg, intradermal), 15 min prior to epinephrine. In this group of rats, stimulus-induced enhancement of epinephrine hyperalgesia was abolished, without affecting epinephrine hyperalgesia (Fig. 2, n = 8/group).

Beta$_2$-adrenergic receptor antagonism

Beta-blockers, such as propranolol, which are antagonists at both β_1 and β_2-adrenergic receptors, are amongst the most commonly used drugs for the treatment of migraine headache (Firnhaber and Rickett, 2009, Supiot, 2009), suggesting that tonic activity at β-adrenergic receptors contributes to the predisposition to migraine. In support of this hypothesis, stress is generally recognized as the most common antecedent to a migraine attack (Martin et al., 2005, Tuncel et al., 2008).

We found that the β_2-adrenergic receptor selective antagonist, ICI-118,551, attenuated stimulus-induced enhancement of endothelin-1 hyperalgesia (Fig. 3 A, n = 8/group), which is compatible with the suggestion that tonic β_2-adrenergic receptor activity in endothelial cells contributes to peripheral pain mechanisms. While its effect was limited to abolishing the stimulus-induced enhancement of ET-1 hyperalgesia, ICI-118,551 significantly inhibited both epinephrine-induced hyperalgesia and stimulus-induced enhancement of epinephrine hyperalgesia (Fig. 3 B, n = 12/group).

$5HT_{1B}$ and $5HT_{1D}$ receptor agonism

While the effect of triptans on migraine headache is thought to be due to their action on the central and peripheral terminals of the primary afferent nociceptor, which contain $5HT_{1B}$ and $5HT_{1D}$ receptors (Hargreaves and Shepheard, 1999, Classey et al., 2010), and/or at sites in the central nervous system (Goadsby, 1998), these receptors are also present on endothelial cells (Seager et al., 1992, Kilbourne and Winneker, 2001, van den Broek et al., 2002). Therefore, we tested the hypothesis that triptans inhibit stimulus-induced enhancement of hyperalgesia produced by the potent vasoconstrictor, endothelin-1 and the β-adrenergic receptor agonist epinephrine. Intradermal injection of sumatriptan (5 μg), 15 min before endothelin-1 (100 ng), not only inhibited stimulus-induced enhancement of endothelin-hyperalgesia, but also endothelin-hyperalgesia (Fig. 4 A). And while sumatriptan also inhibited stimulus-induced enhancement of epinephrine-induced hyperalgesia, it had no effect on epinephrine-induced mechanical hyperalgesia (Fig. 4 B). Triptans also failed to inhibit the mechanical hyperalgesia induced by the prototypical direct-acting pronociceptive inflammatory mediator, prostaglandin E_2 (100 ng) (Fig. 4 C). Thus, triptans may produce antinociceptive effects at least in part, by acting at their cognate receptors located on endothelial cells, as well as on the primary afferent nociceptor.

DISCUSSION

Migraine headache is a complex disorder that likely involves central nervous system and peripheral nervous system, as well as vascular mechanisms (Kurth, 2007, Elliott, 2008, Levy, 2012, McCrory and Gray, 2012). A role of neurovascular mechanisms in migraine headache has been suggested based on a number of clinical observations, including the occurrence of vasoconstriction followed by vasodilatation at onset of migraine headaches, decrease in migraine pain with compression of the temporal artery, and precipitation of migraine headache with vasodilators. Furthermore, treatment of migraine with vasoconstrictors and beta-blockers, association of migraine with vascular diseases (e.g., systemic lupus erythematous, patent foramen ovale, angina, Raynaud's and A–V malformation), and increase in plasma endothelin level at onset of migraine (Watts et al., 1995, Edvinsson et al., 2005), are all compatible with neurovascular involvement in migraine. While more recent studies provide evidence for an important role of the central and peripheral nervous system in migraine headache (Dodick and Silberstein, 2006, Goadsby, 2012, Samsam, 2012), many of these studies also support the suggestion that there is an interaction between the peripheral nervous system and blood vessels (May, 2003, Olesen et al., 2009). While the peripheral contribution has, in general, been related to primary afferent nociceptor function (Shepard et al., 1991), abnormalities in vascular function have also been described (May, 2003). However, how altered vascular function relates to the primary manifestations of migraine headache has not been established. In the present experiments we tested the hypothesis that the representatives of two major classes of drugs used in the treatment of migraine headache inhibit endothelin or epinephrine hyperalgesia, at least in part, by action on endothelial cells.

In a previous study we established that vascular endothelial cells play a role in pain induced by the potent vasoconstrictor peptide, endothelin-1 (Joseph E.K. et al., 2012 in review). We

hypothesized that drugs used in the treatment of migraine headache would inhibit the endothelial cell contribution to endothelin-hyperalgesia. Therefore, we evaluated the effect of two major classes of compounds, a β-adrenergic receptor antagonist, ICI-118551 and a $5HT_{1B}$ and $5HT_{1D}$ agonist, sumatriptan, both of which have receptors in endothelial cells, on endothelin hyperalgesia. Both compounds blocked stimulus – induced enhancement of endothelin hyperalgesia, while alone having no effect on nociceptive threshold. Since ICI-118,551 has inverse agonist properties, it could operate to suppress signaling, in part, absent an endogenous ligand, but still presumably by action on the endothelial cell. Furthermore, sumatriptan failed to attenuate the hyperalgesia induced by the direct-acting pronociceptive inflammatory mediator, prostaglandin E_2, compatible with a role of endothelial cells in vascular pain.

That endothelial cells may play a role in migraine headache raises some interesting questions. For example, one of the diagnostic tests for migraine headache has been the precipitation of a headache by the administration of nitroglycerin, a potent vasodilator, which increases nitric oxide (NO) in the endothelial cell (Greco et al., 2011). One of the unusual characteristic features of the nitroglycerin provocation test is that in patients with migraine headache, there is a distinct delay from the administration of nitroglycerin and the onset of the migraine headache (Schoonman et al., 2006). We suggest that such a delay might be explained by an indirect mechanism of action, such as by its action to increase NO in endothelial cells, leading to the release of mediators that act on nociceptors to produce headache pain. A second interesting clinical correlation relates to the suggestion that triptans are considered specific for migraine headache (Pym et al., 1991, Classey et al., 2010). In support of this, we observed that sumatriptan did not treat prostaglandin-induced mechanical hyperalgesia. Since the receptors on which the triptans are thought to act, $5HT_{1B}$ and $5HT_{1D}$ are present on dorsal root ganglion as well as trigeminal ganglion neurons (Pierce et al., 1996, Classey et al., 2010), triptans may be effective against non-trigeminal vascular pain, by action at both the nociceptors and endothelial cells, and potentially at other levels of the neuraxis.

In a previous study (Joseph EK et al, 2012, in review), we tested the hypothesis that ATP functions as a mediator of stimulus-induced enhancement of hyperalgesia using A-317491, a P2X3 antagonist. We found that A-317491 significantly inhibit stimulus-induced enhancement of hyperalgesia for ET-1. Therefore, a similar hypothesis was tested in this study for stimulus-induced enhancement of hyperalgesia for epinephrine. We found that A-317491 markedly inhibited epinephrine induced SIEH. Endothelial cells lining the vessel lumen are constantly subjected to conditions of varying blood flow and shear stress. The release of vasoactive substances including ATP during conditions of increased shear stress has been extensively documented (Bodin and Burnstock, 1995). A mechanical stimulus (stress) may cause the vesicular release of ATP, which may be responsible for the enhancement of hyperalgesia. However, further research is required to determine the exact mechanisms responsible for this event.

Of note, we found that triptans not only prevented stimulus-induced enhancement of endothelin-1 hyperalgesia but also endothelin-1 hyperalgesia, while the beta-blockers only inhibited stimulus-induced enhancement of endothelin-1 hyperalgesia. This additional effect

of the triptans may help to explain the notable efficiency of the triptans for the treatment of migraine headache.

In conclusion, we provide evidence that anti-migraine compounds can produce anti-hyperalgesia by action on endothelial cells. And, while the role of this particular mechanism in the treatment of migraine headache remains to be established, a better understanding of the role of endothelial cells in vascular pain may help identify novel targets for the treatment of migraine headache as well as other vascular related pain syndromes.

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Highlights

- Endothelin-1 and epinephrine induce mechanical hyperalgesia.
- Mechanical stimulation enhances endothelin-1 and epinephrine-induced hyperalgesia.
- Such enhanced hyperalgesia is dependent on P2X3 receptor activation.
- Triptan and beta-blocker attenuate stimulus-induced enhancement of hyperalgesia.

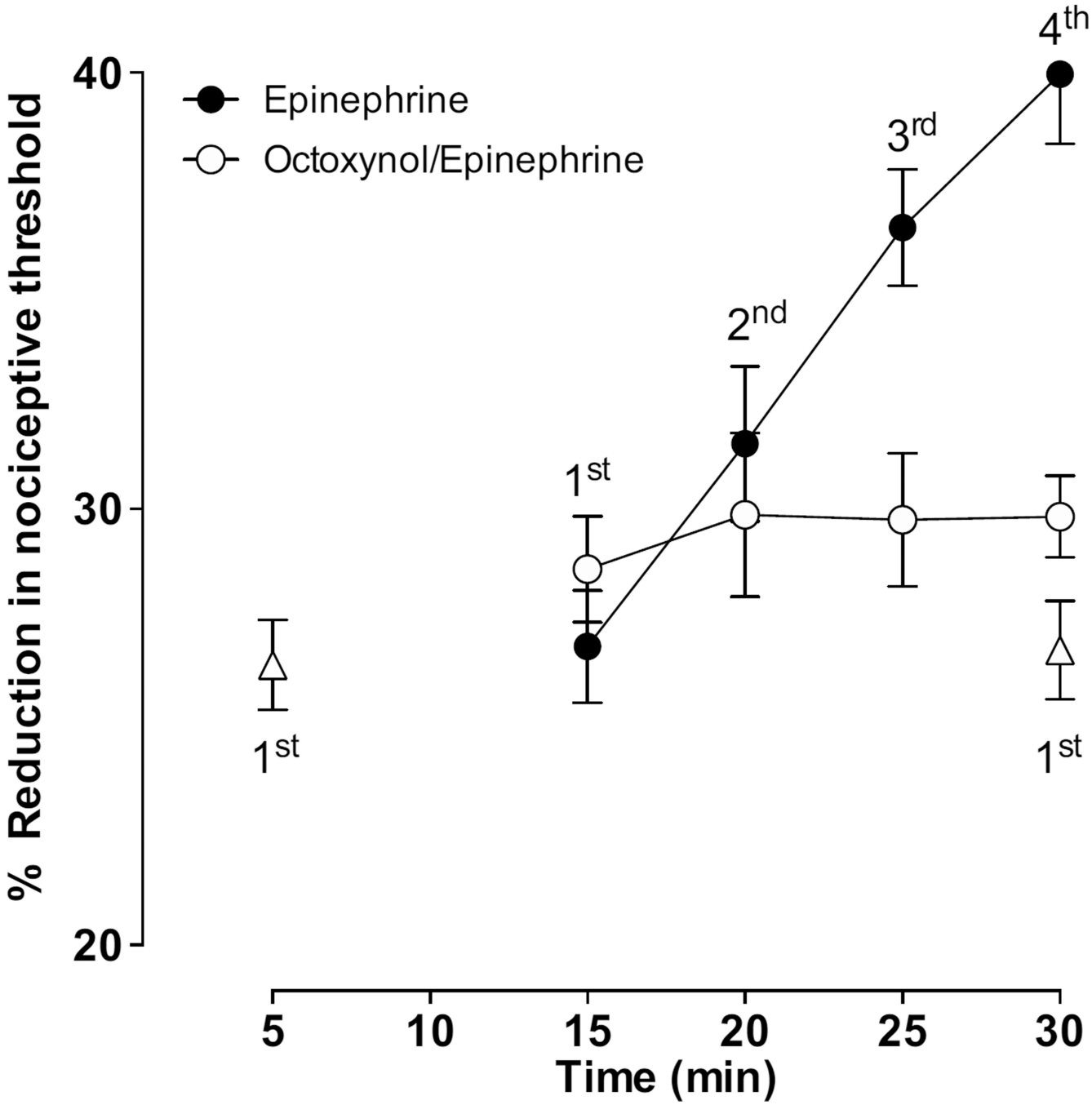

Figure 1. Effect of octoxynol-9 on epinephrine-induced mechanical hyperalgesia and stimulus-induced enhancement of epinephrine hyperalgesia

Intradermal administration of epinephrine (100 ng) produced mechanical hyperalgesia ($P < 0.001$, $n = 8$). Hyperalgesia reached at 5'after epinephrine administration (single reading) was similar to that observed after a single reading at 30' measured in a separate group of rats (triangular symbols). Repeated readings (15–30') of paw withdrawal thresholds at 5 min intervals, caused enhancement of hyperalgesia (further decrease in paw withdrawal threshold on repeated application of the threshold stimulus). Intravenous administration of octoxynol-9 (0.5%, V/V, 1 ml/kg) 15 min prior to epinephrine, abolished stimulus-induced

enhancement of epinephrine hyperalgesia ($P < 0.001$, two way repeated measures ANOVA followed by Bonferroni post test, comparing results within and between groups at different time points, n = 8/group) without affecting the epinephrine hyperalgesia.

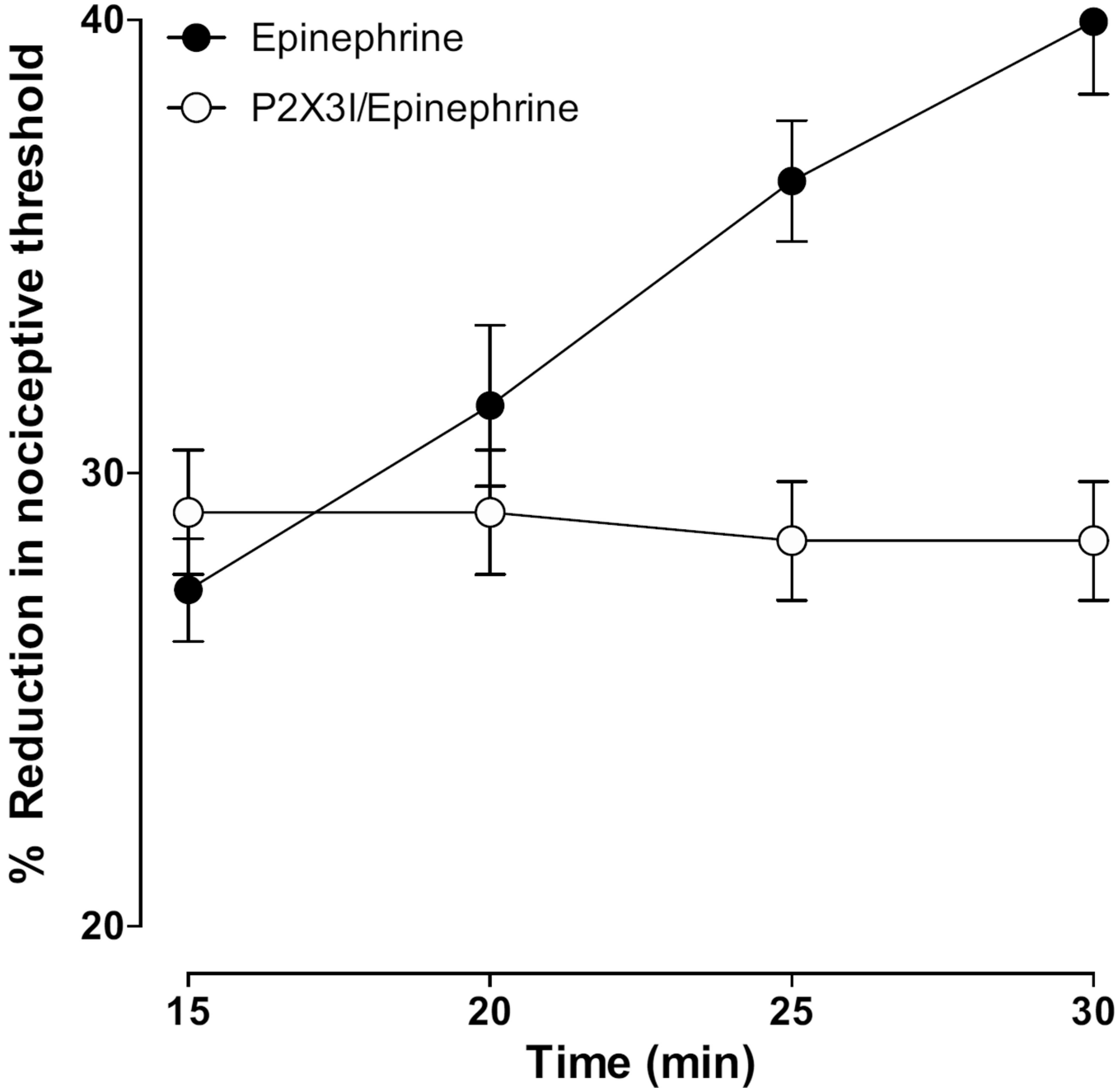

Figure 2. Effect of A-317491 (P2X2/3 inhibitor) on epinephrine-induced mechanical hyperalgesia and stimulus-induced enhancement of epinephrine hyperalgesia

Intradermal administration of A-317491 (1 μg/paw) 15 min prior to epinephrine, abolished stimulus-induced enhancement of epinephrine hyperalgesia ($P < 0.001$, two way repeated measures ANOVA followed by Bonferroni post test, comparing results within and between groups at different time points, n = 8/group) without affecting the epinephrine hyperalgesia.

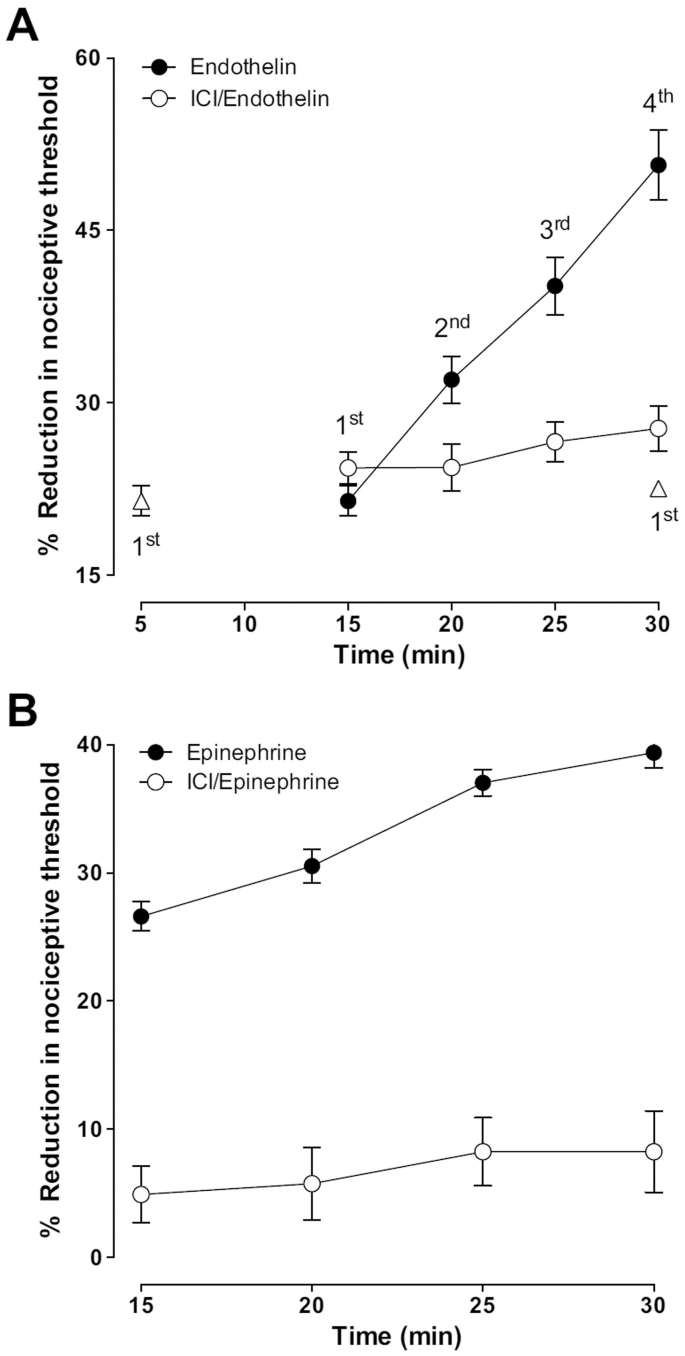

Figure 3. Effect of ICI 118,551 (β_2 - selective adrenergic antagonist) on endothelin-1 (A) and epinephrine (B) induced mechanical hyperalgesia and stimulus-induced enhancement of their hyperalgesia

Intradermal administration of ICI 118,551 (1 μg/paw) 15 min prior to endothelin, abolished stimulus-induced enhancement of endothelin hyperalgesia (**A**, $P < 0.001$, two way repeated measures ANOVA followed by Bonferroni post test, n = 8/group) without affecting the endothelin hyperalgesia. Intradermal administration of ICI 118,551 (1 μg/paw) 15 min prior to epinephrine abolished both epinephrine hyperalgesia and stimulus-induced enhancement of hyperalgesia (**B**, for both $P < 0.001$, two way repeated measures ANOVA followed by

Bonferroni post test, comparing results within and between groups at different time points, n = 12/group).

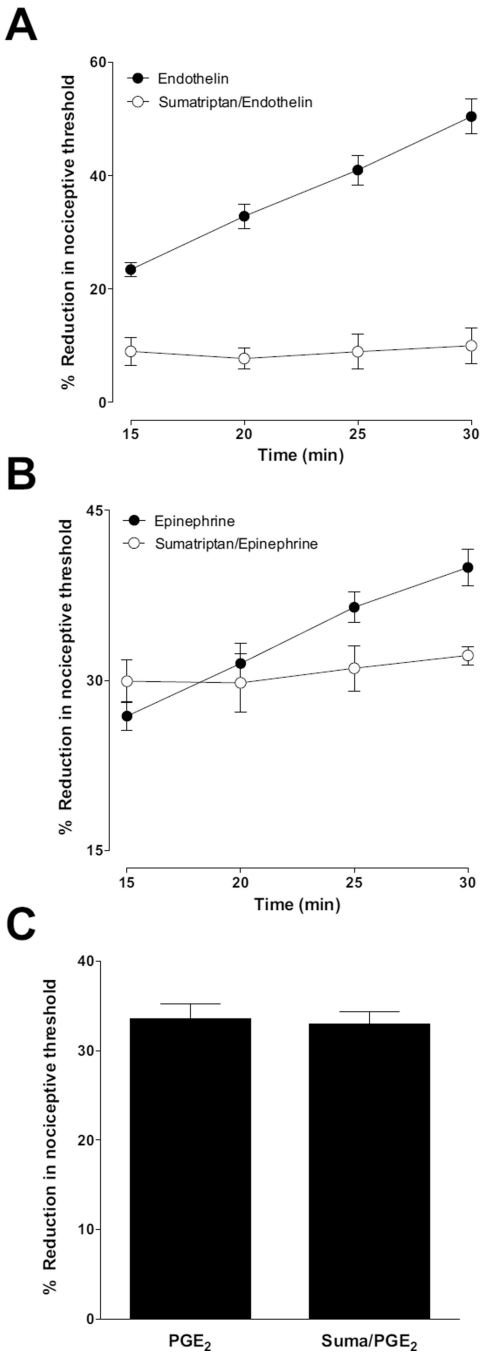

Figure 4. Effect of sumatriptan (5-HT$1_{B/D}$ agonist) on endothelin-1 (A), epinephrine (B) induced mechanical hyperalgesia and stimulus-induced hyperalgesia and on PGE_2 (C) induced mechanical hyperalgesia

Intradermal administration of sumatriptan (5 μg/paw) 15 min prior to endothelin, attenuated endothelin hyperalgesia and stimulus-induced enhancement of endothelin hyperalgesia (**A**, for both $P < 0.001$, two way repeated measures ANOVA followed by Bonferroni post test, n = 8/group), whereas sumatriptan treatment 15 min prior to epinephrine, only attenuated stimulus-induced enhancement of epinephrine hyperalgesia (**B**, $P < 0.001$, two way repeated measures ANOVA followed by Bonferroni post test, comparing results within and between

groups at different time points, n = 8/group). Intradermal administration sumatriptan 15 min prior to PGE_2 (100 ng) had no effect on PGE_2 hyperalgesia (**C**, n = 6).