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Norepinephrine-induced nociception and vasoconstrictor hypersensitivity in rats with chronic post-ischemia pain

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Abstract

Painful hypersensitivity to norepinephrine (NE) has been reported in various chronic pain conditions that exhibit sympathetically-maintained pain (SMP), particularly CRPS-I and II. We investigated the parallels between the nociceptive and vascular sensitivity to NE in rats with chronic post-ischemia pain (CPIP), an animal model of CRPS-I induced by hind paw ischemia–reperfusion injury. Intradermal injections of NE to the affected hind paw induced dose-dependent nociceptive behaviours in CPIP rats, but not sham animals. These behaviours were blocked by α_1- and α_2-adrenergic receptor antagonists, or a nitric oxide (NO) donor. Using laser Doppler flowmetry, we detected vasoconstrictor hypersensitivity in the ipsilateral CPIP hind paw, as compared to responses in sham animals or the contralateral hind paw. The vasoconstrictor hypersensitivity was also attenuated by adrenergic antagonists. Intradermal injection of [Arg^8] vasopressin (AVP) or the endothelial NO synthase (eNOS) inhibitor, L-NIO, to the affected paw also induced nociceptive behaviours in CPIP rats, but not sham rats. These results suggest CPIP rats display abnormal nociceptive responses to adrenergic and non-adrenergic vasoconstrictive agents. Furthermore, the nociceptive responses to NE in CPIP rats are paralleled by enhanced vasoconstrictive responses to NE, and are relieved by α-adrenergic antagonists or a vasodilator. We conclude that persistent tissue ischemia and hypersensitivity to sympathetic vasoconstriction are important mechanisms for pain in CPIP rats, and that either reducing vasoconstriction or enhancing vasodilatation may be effective methods of relieving the pain of CRPS-I.

Keywords: Chronic post-ischemia pain; Adrenergic receptors; Ischemia; Laser Doppler; Vasoconstriction; [Arg^8] vasopressin; L-NIO; Sodium nitroprusside; SIN-1; Complex regional pain syndrome; CRPS-I

1. Introduction

Intradermal injection of NE normally produces a burning, stinging pain that lasts tens of seconds. In patients with CRPS-I or II, intradermal NE [58], or the α_1-adrenergic agonist phenylephrine [36a], produces the same transient pain, but also abnormal burning pain and mechanical allodynia lasting for tens of minutes. Abnormal responses to NE or phenylephrine are also found in CRPS-I and II patients who have first had their pain relieved by surgical sympathectomy, sympathetic ganglion blocks or intradermal treatment with phentolamine or clonidine [2,17,58,61]. The ability of intradermal NE to rekindle [58] pain relieved by

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sympathectomy, or sympathetic blocks, suggests that the pain depends more on hyper-responsiveness of adrenoceptors than on hyperactivity of sympathetic fibers.

The hypersensitivity to NE, along with the reduction of pain and allodynia in some CRPS-I and II patients with sympathetic blocks or adrenergic antagonists, has been taken as evidence for sympathetically-maintained pain (SMP) [10,47]. Importantly, exaggerated painful responses to NE are exhibited in CRPS-I and II patients whose pain is relieved by sympathetic blocks (i.e., SMP), but not in those whose pain is not relieved by sympathetic blocks or phentolamine (sympathetically-independent pain, SIP) [2,36a,58].

While experimental evidence has suggested that SMP may depend on sympathetic efferent–primary afferent coupling [12] that causes *de novo* adrenergic sensitivity in damaged afferents [18,60], dorsal root ganglion cells [19,39] or nociceptors [43,49], clinical evidence further suggests that SMP may depend on sympathetic-dependent vasoconstriction that produces pain by reducing blood flow in the affected tissue [1,5,30,64]. Indeed, NE-induced pain in CRPS patients occurs at doses which produce vasoconstriction [2], and CRPS-I patients show a hyper-responsiveness of vascular responses to NE [3,8,55]. Furthermore, it has been shown that there is enhanced vasoconstriction to exogenous NE following chronic constriction injury (CCI) of the sciatic nerve (an animal model of CRPS-II) [31,32]. Recently, our group developed a novel animal model of chronic post-ischemia pain (CPIP) that is created by a 3-h hind paw tourniquet ischemia and displays persistent mechanical and cold allodynia as a result of an ischemia–reperfusion (I–R) injury [15,33]. The purpose of this study is to examine the relationship between NE-evoked nociception and vasoconstrictor hypersensitivity (as reflected by NE-induced changes in skin blood flow) in this animal model which may be particularly relevant to CRPS type I.

2. Methods

2.1. Animals

Male Long Evans hooded rats (275–325 g, Charles River, St. Constant, Que., Canada) were housed in groups of 3–4, with food and water available *ad libitum*, on a 12:12 h light:-dark cycle. All treatments and testing procedures were approved by the Animal Care Committee at McGill University, and conformed to the ethical guidelines of the Canadian Council on Animal Care and the International Association for the Study of Pain.

2.2. Animal model of CRPS-I

Chronic post-ischemia pain (CPIP) was generated following exposure to prolonged hind paw ischemia and reperfusion as described in Coderre et al. [15]. Briefly, rats were anesthetised over a 3 h period with a sodium pentobarbital infusion. After induction of anesthesia, a Nitrile 70 Durometer O-ring (O-rings West, Seattle, WA, USA) with 7/32″ internal diameter was placed around the rat's left hind limb just proximal to the ankle joint. The termination of sodium pentobarbital anesthesia was timed so that rats recovered fully within 30–60 min following reperfusion, which occurred immediately after removal of the O-ring. Sham rats received exactly the same treatment, except that the O-ring was cut so that it only loosely surrounded the ankle, and did not occlude blood flow to the hind paw.

2.3. Drugs

Norepinephrine bitartrate dihydrate (NE), yohimbine, prazosin and sodium nitroprusside (SNP) were all obtained from Sigma–Aldrich (St. Louis, MO, USA). [Arg^8] Vasopressin (AVP) was obtained from Calbiochem (La Jolla, CA, USA). 3-morpho-linylsydnoneimine chloride (SIN-1) and *N*5-(1-iminoethyl)-L-ornithine dihydrochloride (L-NIO) were obtained from Tocris (Bristol, UK). All agents were diluted in a 0.9% saline vehicle immediately before experiments.

2.4. Nociceptive testing

2.4.1. Mechanical sensitivity

Hind paw mechanical thresholds were assessed by measuring the withdrawal response to von Frey filament stimulation according to a modification of the up/down method described by Chaplan et al. [11]. In brief, animals were placed in a Plexiglas® box ($21 \times 16 \times 27$ cm^3) with a wire grid bottom through which the von Frey filaments (nylon monofilaments; Stoelting, Woodale, IL, USA) were applied to the plantar surface of the hind paw. Filaments were applied in either ascending or descending strength as necessary to determine the filament closest to the threshold of response. Each filament was applied once for 10 s to the center of the paw between the digital tori. A lower intensity hair followed each positive response and a higher intensity hair followed each negative response until five responses were recorded after a first change in response. The minimum filament intensity was 0.25 g and the maximum was 15 g. Based on the response pattern and the force of the final filament, the 50% response threshold (grams) was calculated. The resulting pattern of positive and negative responses was tabulated, and the 50% response threshold was interpolated using the formula: 50% g threshold $= (10^{[x_f + k\delta]})/10{,}000$, where x_f is the value (in log units) of the final von Frey hair used; k is tabular value (see [11] for pattern of positive/negative responses); and δ is the mean difference (in log units) between stimuli (here 0.224). Hairs were from the standard Semmes–Weinstein series [53]. Mechanical sensitivity was tested before CPIP induction, and 2 and/or 7 days post-reperfusion. Animals were classified prior to experiments as responders if their von Frey paw withdrawal scores were below 6 g (65.4%) and non-responders if their paw withdrawal threshold scores were above 10 g (27.3%). Animals with von Frey scores between 6 and 10 g (7.3%) were not used.

2.4.2. Evoked nociceptive behaviours

To measure pain evoked by intradermal injections, rats were placed in Plexiglas® boxes with a mirror underneath in order to observe nociceptive behaviours. Rats were habituated to the testing apparatus 30 min each day for 2–3 days prior to testing and for a minimum of 30 min immediately prior to testing. Drugs were injected in volumes of 20 μl to the plantar surface of the hind paw using a 26 G needle. Two injected rats were then observed simultaneously for 15 min, and the total time spent exhibiting hind paw stamping, elevation or licking was recorded. Experiments were performed in blocks with groups of sham rats, CPIP rats and CPIP non-responder rats tested on the same days. Rats were only used for one experiment, and at the time of testing the experimenter was blind to the animal's treatment.

In the first behavioural experiment, we assessed whether saline vehicle, or 10, 50 or 250 ng of intraplantar NE-induced nociceptive behaviours in sham or 2- or 7-day CPIP responders and non-responders ($n = 6$–9/group). In subsequent experiments, adrenergic antagonists (0.5, 2 and 10 μg of prazosin and yohimbine) were co-injected with 250 ng NE to determine which adrenoceptors contributed to the NE-induced behaviours in 2-day CPIP rats ($n = 6$/group). The prazosin and yohimbine doses used here have been shown to relieve mechanical hyperalgesia in rat models of neuropathic pain [46,59]. Additional studies assessed whether NE-induced pain behaviours in 2-day CPIP rats were attenuated by intraplantar or systemic administration of NO donors. SNP was co-injected with 250 ng NE at doses of 20, 100 and 500 μg ($n = 6$/group), and systemic SIN-1 (10 m/kg) or vehicle ($n = 6$–8) was used as a pretreatment prior to 250 ng intraplantar NE. The doses of the NO donors used here have previously been shown to reduce allodynia in a rat model of inflammatory pain [16], and do not produce motor or sedative side effects [66]. Finally, we examined whether intraplantar injections of a non-adrenergic vasoconstrictor, AVP (500 ng), or an NO synthase inhibitor, L-NIO (250 μg), ($n = 7$–10 per group) induced nociceptive behaviours in sham or 2-day CPIP rats.

2.5. Laser Doppler flowmetry

Laser Doppler flowmetry was used to assess blood flow changes associated with either close arterial or intravenous injection of NE. After behavioural testing for von Frey thresholds, animals were anesthetised with urethane (1 mg/kg, i.p.), and their body temperature was maintained at 37 °C using a thermostatically-controlled heating pad coupled to a rectal probe (Stoelting, IL, USA). For close arterial injections of NE, the common iliac artery was exposed on the side contralateral to the CPIP injury. The artery was catheterized with PE-10 tubing filled with warm saline (37 °C) and aimed upwards towards the aortic bifurcation so that solutions were injected immediately upstream of the common iliac artery on the CPIP side. The wound was covered with saline-soaked gauze. A laser Doppler probe (DP1T-V2; Moor Instruments, Axminster, UK) with a height of 12.5 mm, outer diameter of 8 mm, and a fiber separation of 0.5 mm was taped loosely on the plantar surface of the ipsilateral paw adjusting the position and pressure to give initial readings of approximately 150–300 arbitrary flux units. Blood flow was recorded continuously with a DRT4 monitor (Moor Instruments, Axminster, UK). Animals were covered with a blanket and left undisturbed for approximately 60 min in order to obtain stable baseline for flux, as well as hind paw and core body temperatures. Animals whose baseline arbitrary flux units were below 150 were not used.

Since close arterial injection necessitated cannulating and compromising blood flow in the common iliac artery contralateral to the CPIP injury, it was not possible in these experiments to compare NE-induced changes in blood flow in the ipsilateral and contralateral hind paws. In order to make this comparison, we also performed laser Doppler flowmetry following intravenous administration of NE to the jugular vein. The jugular vein was catheterized with PE-20 tubing, and two separate laser Doppler probes were used to simultaneously record the ipsilateral and contralateral hind paw blood flow in CPIP rats.

2.5.1. NE-evoked vasoconstriction

All drugs and saline were warmed to 37 °C and 20 μl of drug/vehicle was injected followed by an 80 μl saline flush. An initial saline (control) injection was given after establishment of a steady baseline. No flow disturbance was observed, and subsequently, ascending doses of NE (50, 100 and 200 μg/kg) were administered every 20 min to naïve ($n = 7$), sham ($n = 13$), 2-day CPIP responders ($n = 8$), 7-day CPIP responders ($n = 9$), 2-day CPIP non-responders ($n = 9$), and 7-day CPIP non-responders ($n = 7$). Dose–response effects of close arterial NE injections were also examined in rats that were pretreated intra-arterially with 10 μg of either prazosin or yohimbine ($n = 8$ per group). The same doses of NE that were used for close-arterial injections were used in another experiment to compare the effect of intravenous NE on blood flow changes in the ipsilateral and contralateral hind paws of sham ($n = 9$) and 2-day CPIP responders ($n = 9$). Rats were only used once and were sacrificed at the end of the experiment with an overdose of sodium pentobarbital.

2.5.2. Flowmetry calculations

Baseline flux was defined as the average flux value in the 3 min prior to the initial injection (saline). Peak decreases in blood flow, expressed as % change from baseline, were calculated using the minimum flux value after each NE injection. Since arbitrary flux values are dependent on depth of anesthesia and laser Doppler probe placement, this study assessed only NE-induced changes in arbitrary flux values, and did not compare differences in basal flux values between groups. Although reduced basal flux might be expected after CPIP, and we have previously shown that there is reduced perfusion in muscle of the ipsilateral hind paw [33], we did not here observe ipsilateral/contralateral differences in baseline flux values in CPIP rats.

2.6. Statistics

Group comparisons of NE-evoked nociceptive scores were performed using two-way ANOVA followed by Fisher's LSD tests. Group comparisons for laser Doppler peak flux decreases were analyzed using two-way repeated measures

ANOVA and Fishers LSD test. One-way ANOVA was used to compare the behavioural effects of SNP vs. vehicle and AVP vs. vehicles.

3. Results

3.1. NE-induced nociceptive responses

Approximately 70% of animals subjected to the I–R injury displayed mechanical allodynia (von Frey threshold below 6 g) and were classified as responders. This is consistent with our past studies as reported in Coderre et al. [15].

Fig. 1A shows the time spent exhibiting vehicle- and NE-evoked nociceptive behaviours in sham rats at 2 days and CPIP rats (responders) at 2 and 7 days post-reperfusion. Nociceptive behaviours generally started a few seconds after injection and peaked between 2 and 5 min post-injection. The most common behaviours were hind paw stamping, elevation and licking. Two-way ANOVA revealed a significant main effect of group ($F_{2,89} = 16.53$, $P < 0.0001$), and dose ($F_{3,89} = 9.05$, $P < 0.0001$) and a significant group × dose interaction ($F_{6,89} = 2.31$, $P < 0.05$) for animals given intradermal injections of vehicle, 10, 50 or 250 ng of NE. The vehicle injection did not produce significantly greater nociceptive behaviours in CPIP rats than in sham rats. However, at 2 days post-perfusion, CPIP rats given either 50 ($P < 0.05$) or 250 ng ($P < 0.01$) of NE exhibited significantly more nociceptive behaviours than CPIP rats given vehicle. For the CPIP rats at 7 days post-reperfusion, the 50 and 250 ng doses of NE also produced significantly more nociceptive behaviours ($P < 0.01$) as compared to vehicle in CPIP rats.

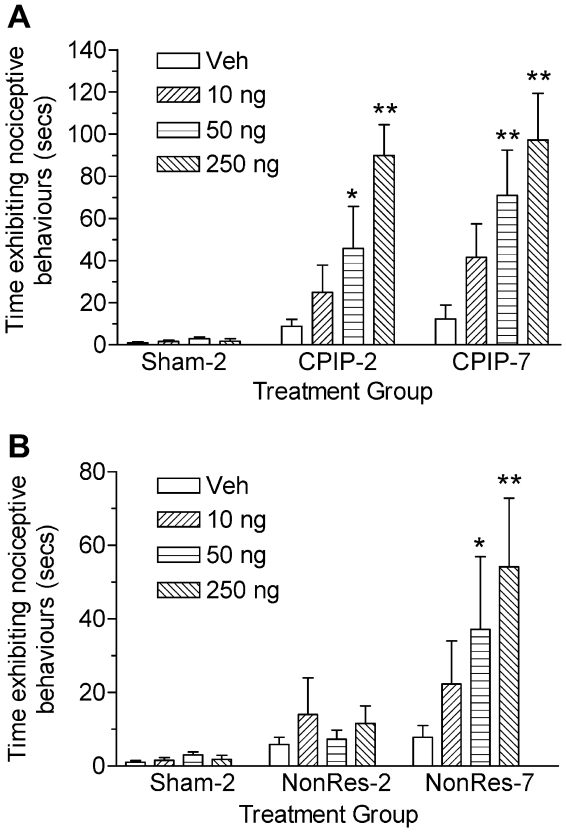

Fig. 1. (A) Intradermal NE-evoked nociception in CPIP responders at 2 and 7 days post-reperfusion. CPIP responders displayed exaggerated NE-induced nociception with the 50 and 250 ng NE doses on days 2 and 7. ($^{*}P < 0.05$ and $^{**}P < 0.01$). (B) Intradermal NE-evoked nociception in CPIP non-responders at 2 and 7 days post-reperfusion. CPIP non-responders displayed exaggerated NE-induced nociception with the 50 and 250 ng NE doses on day 7 only. ($^{*}P < 0.05$ and $^{**}P < 0.01$)

Fig. 1B shows the time spent exhibiting vehicle- and NE-evoked nociceptive behaviours in sham rats and CPIP *non-responders* at 2 and 7 days post-reperfusion. Two-way ANOVA revealed a significant main effect of group ($F_{2,80} = 11.87$, $P < 0.0001$), but a non-significant main effect of dose, and a non-significant group × dose interaction. Post hoc analysis revealed that once again sham rats did not exhibit greater response to any dose of NE relative to the vehicle, and CPIP non-responders did not exhibit significantly more nociceptive behaviours to vehicle than did sham rats. At 2 days post-reperfusion, none of the NE doses induced significantly more nociceptive behaviours in CPIP non-responder as compared to CPIP non-responder rats injected with vehicle. However, at 7 days post-reperfusion, the 50 and 250 ng doses of NE induced significantly more nociceptive behaviours in CPIP non-responders as compared to vehicle injections in CPIP non-responders ($P < 0.05$ for the 50 ng dose and $P < 0.01$ for the 250 ng dose).

3.2. Hind paw blood flow responses to close arterial NE

Fig. 2 shows a sample trace for the laser Doppler protocol. The trace shows that flux levels are not altered in response to saline injection, but are lowered between 100 and 300 U for durations that increase with greater intra-arterial doses of NE between 50 and 200 μg/kg.

Baseline arbitrary flux values were not significantly different between naïve, sham, CPIP 2-day responders, CPIP 7-day responders, CPIP 2-day non-responders and CPIP 7-day non-responders. There were no significant group differences between naïve and shams in their NE-evoked decreases in blood flow, so these groups were combined into a single control group. Although a range of doses were tested to elicit significant detectable decreases in flux values, we found that doses below 50 μg/kg showed inconsistent flux decreases in some animals, while doses greater than 200 μg/kg were subject to significant ceiling effects. The peak response was usually seen approximately 1–2 min post-injection, and the return to baseline occurred approximately 7–8 min post-injection for the 50 μg/kg dose, 13–14 min post-

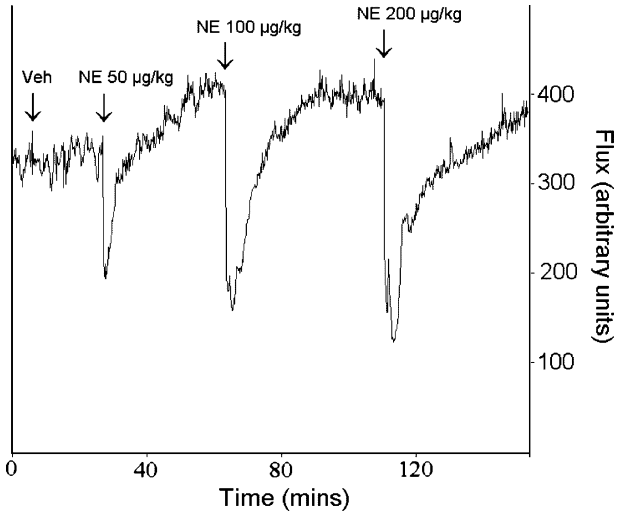

Fig. 2. Sample laser Doppler trace showing an NE dose–response curve in a naïve rat. Intra-arterial saline results in no alteration in blood flow. Intra-arterial NE produces dose-dependent decreases in blood flow (arbitrary flux units).

injection for the 100 μg/kg dose, and usually over 20 min post-injection for the 200 μg/kg (the experiment was stopped 20 min post-injection).

Fig. 3A shows dose–response curves for NE-evoked flux decreases in controls and CPIP rats at 2 and 7 days post-reperfusion. Two-way repeated measures ANOVA demonstrates a significant main effect for group ($F_{2,113} = 5.28$, $P < 0.05$), a significant main effect of dose ($F_{2,113} = 19.94$, $P < 0.01$) but a non-significant group × dose interaction. Except for the 50 μg/kg dose of the CPIP 7-day responder, post hoc analyses reveal that CPIP rats had significantly greater peak decreases in flux compared to controls for all three NE doses at both 2 and 7 days post-reperfusion ($^{*}P < 0.05$, $^{**}P < 0.01$).

Fig. 3B shows dose–response curves for intra-arterial NE-induced decreases in blood flow for controls and CPIP *non-responders* at 2 and 7 days post-reperfusion. ANOVA reveals a significant main effect of dose ($F_{2,107} = 22.22$, $P < 0.0001$), but a non-significant main effect of group, and a non-significant group × dose interaction. These results indicate that NE-induced blood flood decreases did not significantly differ between CPIP non-responders and control rats.

3.3. Effect of intravenous NE administration in CPIP responders

In the animals prepared with jugular vein cannulae, baseline arbitrary flux values were not significantly different between the ipsilateral and contralateral hind paws in CPIP responders ($n = 9$) and shams ($n = 9$). Fig. 3C shows dose–responses curves for intravenous NE-evoked decreases in blood flow for CPIP responders with simultaneous ipsilateral and contralateral hind paw recordings. There is a significant effect of side ($F_{1,53} = 7.27$; $P < 0.05$), a significant effect of dose ($F_{2,53} = 21.13$; $P < 0.0001$), and a non-significant side × dose interaction. Post hoc analyses reveal a significant difference between ipsilateral and contralateral paws with the 50 and 100 μg/kg NE doses ($^{*}P < 0.05$, $^{**}P < 0.01$).

Fig. 3D shows dose–response curves for intravenous NE-evoked decreases in blood flow for shams with simultaneous ipsilateral and contralateral hind paw recordings. Although there was a significant effect of dose ($F_{2,53} = 35.12$; $P < 0.0001$), there was a non-significant effect of side and a non-significant side × dose interaction.

3.4. Effects of adrenergic antagonists on NE-evoked nociception and blood flow decreases in CPIP rats

Fig. 4 demonstrates the effects of the α_1-adrenergic receptor antagonist, prazosin, and the α_2-adrenergic receptor antagonist, yohimbine, on nociceptive behaviours when co-administered intradermally with 250 ng NE at 2 days post-reperfusion. One-way ANOVA reveals a significant main effect of group ($F_{6,41} = 3.99$, $P < 0.01$). Post hoc analyses revealed that co-administration of prazosin at 2 and 10 μg ($^{*}P < 0.05$; $^{**}P < 0.01$), but not 0.4 μg, significantly reduced NE-evoked nociceptive behaviours. Co-administration of 2 and 10 μg ($^{*}P < 0.05$; $^{**}P < 0.01$), but not 0.4 μg, of yohimbine also significantly inhibited NE-evoked nociceptive behaviours.

Fig. 5 shows the effects of 10 μg doses of intra-arterial prazosin and yohimbine on close arterial NE-evoked decreases in blood flow. Two-way repeated measures ANOVA revealed significant main effects of group ($F_{2,71} = 21.65$, $P < 0.0001$) and dose ($F_{2,71} = 21.50$, $P < 0.0001$), as well as a significant group × dose interaction ($F_{4,71} = 3.33$, $P < 0.05$). Post hoc analyses revealed that both prazosin pretreatment significantly reduced the peak decrease in flux at all NE doses ($P < 0.01$), while yohimbine pretreatment significantly reduced the peak decrease in flux at the 50 μg/kg NE dose ($P < 0.01$).

3.5. Effect of co-administration of sodium nitroprusside (SNP) or 3-morpholinylsydnoneimine chloride (SIN-1) pretreatment on NE-evoked nociception in CPIP rats

To further verify whether vasoconstriction may be a mechanism for NE-evoked pain, we tested the effect of intradermal co-administration of NE with SNP, an NO donor and vasodilator, on NE-induced nociceptive behaviours at 2 days post-reperfusion in CPIP responders (Fig. 6A). One-way ANOVA revealed a significant main effect of group ($F_{3,23} = 3.18$, $P < 0.05$). Post hoc analyses revealed a significant inhibition of NE-evoked nociception by the 100 and 500 μg doses of SNP

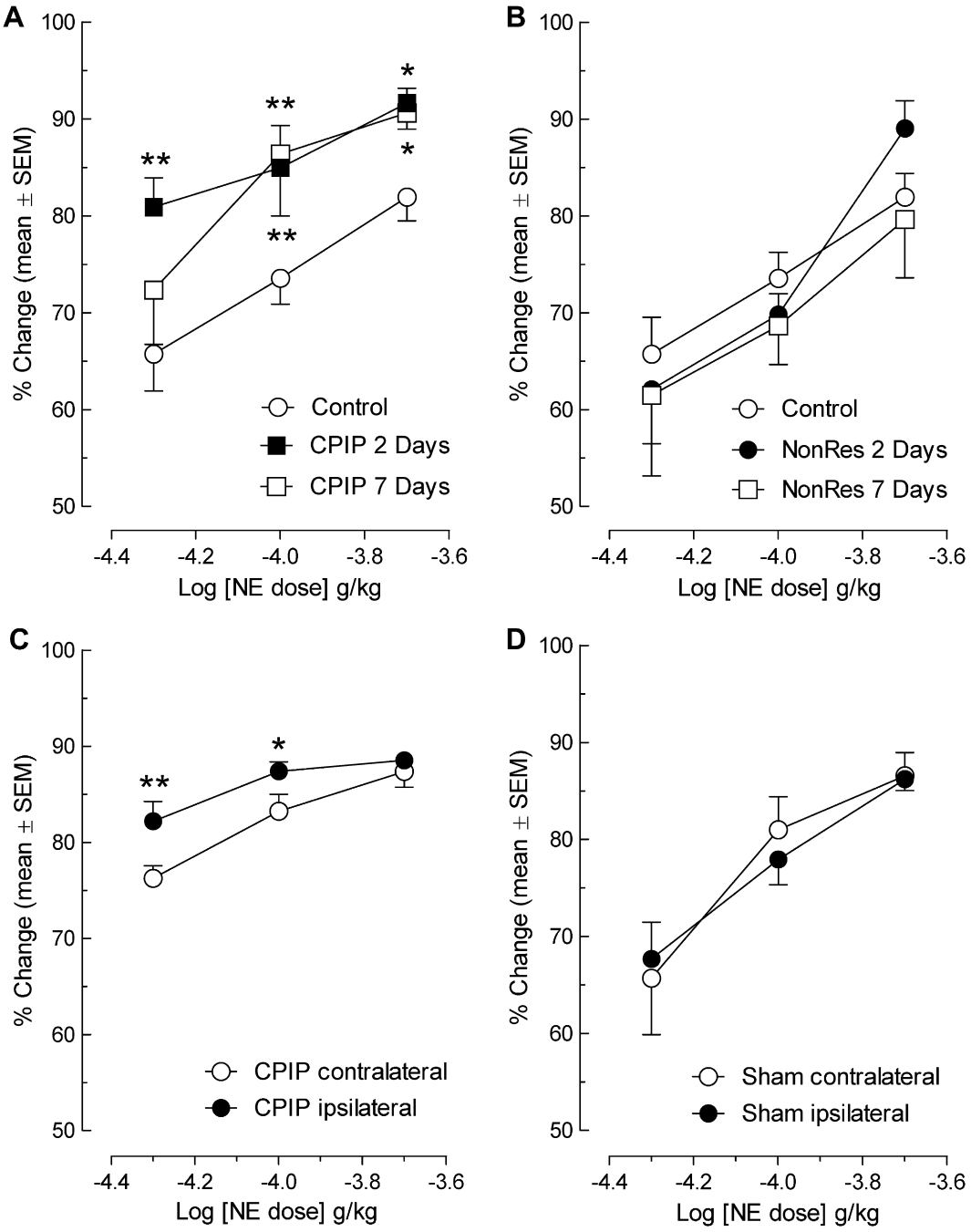

Fig. 3. (A) Intra-arterial NE-evoked decreases in blood flow as determined by % change in the peak response to NE in CPIP responders. CPIP responders show enhanced NE-evoked decreases in blood flow for the 50, 100 and 200 μg/kg NE doses at both 2 and 7 days post-reperfusion ($^{*}P < 0.05$, $^{**}P < 0.01$). (B) Intra-arterial NE-evoked decreases in blood flow in CPIP non-responders. CPIP non-responders do not show enhanced NE-evoked decreases in blood flow for any dose of NE at either 2 or 7 days post-reperfusion. (C) Intravenous NE-evoked decreases in blood flow in CPIP responders. CPIP responders show enhanced NE-evoked decreases in blood flow in the ipsilateral hind paw as compared to the contralateral paw for the 50 and 100 μg/kg NE doses at 2 days post-reperfusion ($^{*}P < 0.05$, $^{**}P < 0.01$). (D) Intravenous NE-evoked decreases in sham control rats. There is no difference in NE-evoked decreases in blood flow between the ipsilateral hind paw as compared to the contralateral paw for any of the NE doses at 2 days post-reperfusion.

($P < 0.05$). We also tested the effect of pretreatment (30 min prior) with an anti-allodynic systemic dose (10 mg/kg) of the NO donor, SIN-1, on intradermal NE-induced nociceptive behaviours at 2 days post-reperfusion (Fig. 6B). One-way ANOVA revealed a significant main effect of group ($F_{2,21} = 6.15$, $P < 0.01$). Vehicle pretreatment followed by 250 ng intradermal NE-induced significant nociceptive behaviours ($P < 0.01$). This effect was attenuated by pretreatment with 10 mg/kg SIN-1 ($P < 0.05$).

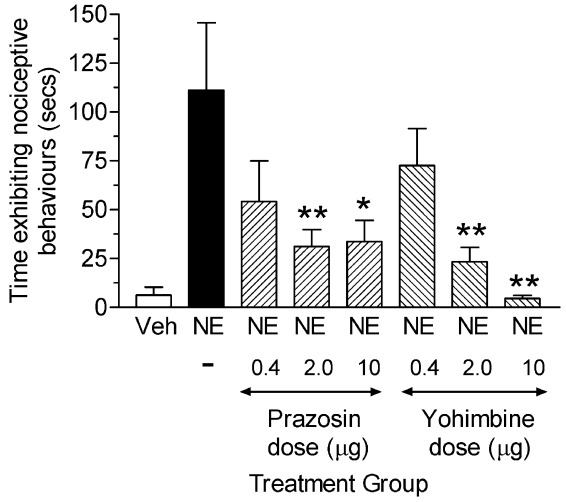

Fig. 4. Effect of intradermal co-administration of α-adrenergic receptor antagonists on NE-induced nociception in CPIP rats. Co-administration of 2 and 10 μg of either prazosin or yohimbine significantly attenuates intradermal NE-evoked nociceptive behaviours in CPIP responders (* $P < 0.05$, ** $P < 0.01$).

3.6. Effect of intradermal administration of a non-adrenergic vasoconstrictor or an eNOS inhibitor in CPIP rats

In a similar fashion as the NE experiments, we examined whether nociceptive behaviours could be induced in 2- and 7-day CPIP responders with an intradermal injection of the non-adrenergic vasoconstrictor, ([Arg^8] vasopressin; AVP), or an eNOS inhibitor, (N5-(1-iminoethyl)-L-ornithine dihydrochloride; L-NIO). Fig. 7A shows the effect of intradermal injection of 500 ng AVP at inducing nociceptive behaviours in CPIP responders as compared to AVP in sham control rats and vehicle injection in CPIP responders at 2 and 7 days post-reperfusion. Two-way ANOVA reveals significant main effects of treatment ($F_{1,44} = 12.48$, $P < 0.01$) and condition ($F_{2,44} = 4.38$, $P < 0.01$), but a non-significant treatment × condition interaction. Vehicle injection in CPIP rats did not produce significant nociceptive behaviours at 2 or 7 days post-reperfusion as compared to vehicle injections in sham rats. AVP (500 ng) induced

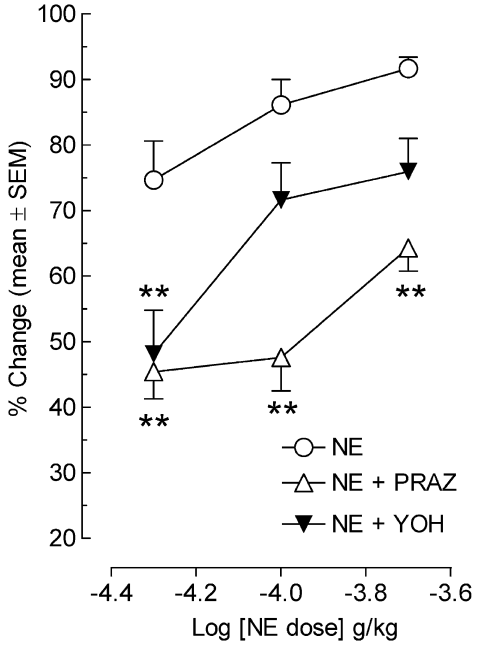

Fig. 5. Effect of intra-arterial pretreatment with α-adrenergic receptor antagonists on intra-arterial NE-induced decreases in blood flow in CPIP responders. Administration of 10 μg prazosin results in an attenuated decrease in blood flow in response to intra-arterial NE at the 50, 100 and 200 μg/kg NE doses. Administration of 10 μg yohimbine results in attenuated decreases in blood flow in response to intra-arterial NE at the 50 μg/kg NE dose only. (** $P < 0.01$).

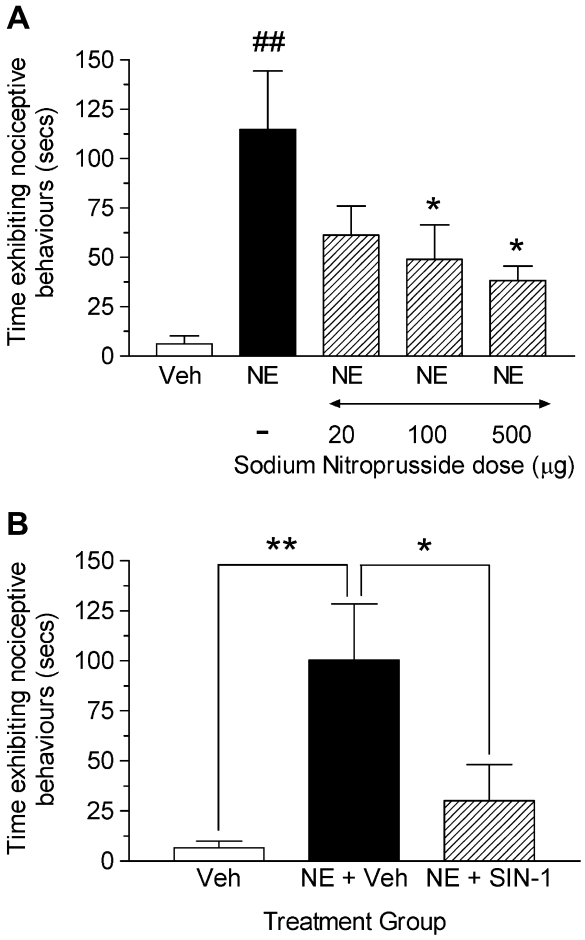

Fig. 6. (A) Effect of intradermal sodium nitroprusside on intradermal NE-induced nociception in CPIP rats. NE alone again produced significantly greater nociceptive behaviours than vehicle (## $P < 0.01$). Co-administration of 100 or 500 μg doses of sodium nitroprusside with 250 ng intradermal NE results in attenuated nociceptive behaviours as compared to NE alone (* $P < 0.05$). (B) Effect of systemic pretreatment with SIN-1 on intradermal NE-induced nociception in CPIP rats. Administration of 10 mg/kg of SIN-1 30 min prior to 250 ng intradermal NE results in attenuated nociceptive behaviours as compared to NE alone (* $P < 0.05$), which was significantly greater than vehicle injection (** $P < 0.01$).

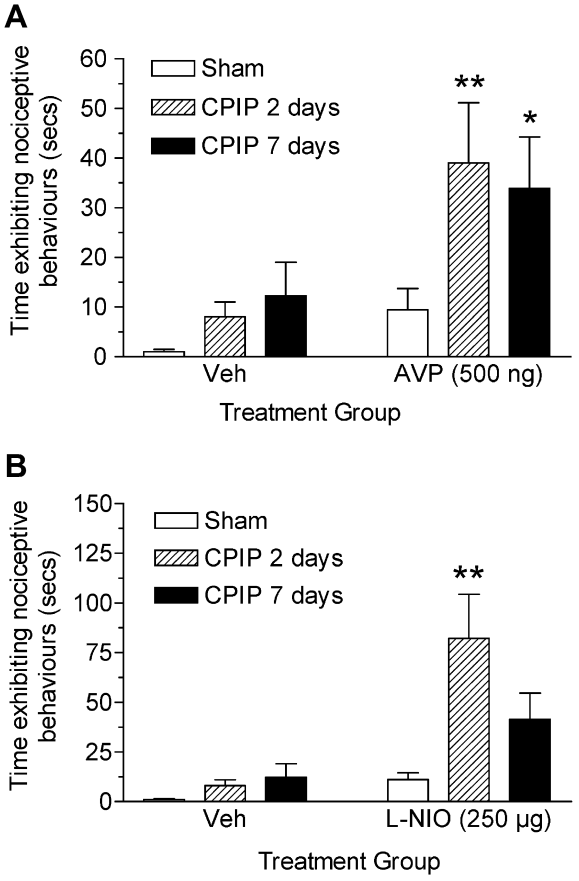

Fig. 7. (A) Nociceptive effects of a non-adrenergic vasoconstrictor, vasopressin. Intradermal administration of 500 ng [Arg^8] vasopressin evokes significant nociceptive behaviours in CPIP rats as compared to vehicle at both 2 and 7 days post-reperfusion ($^*P < 0.05$; $^{**}P < 0.01$). (B) Nociceptive effects of an eNOS inhibitor, L-NIO. Intradermal administration of 250 µg L-NIO evokes significant nociceptive behaviours in CPIP rats as compared to vehicle at 2 days, but not at 7 days post-reperfusion ($^*P < 0.05$ and $^{**}P < 0.01$).

significant nociceptive behaviours at 2 and 7 days post-reperfusion, as compared to vehicle in CPIP responders ($^*P < 0.05$, $^{**}P < 0.01$). AVP did not produce greater nociceptive behaviours in sham controls as compared to vehicle injection.

Fig. 7B shows the effect of intradermal injection of 250 µg L-NIO at inducing nociceptive behaviours in CPIP-responders as compared to L-NIO in sham controls or vehicle in CPIP responders at 2 and 7 days post-reperfusion. Two-way ANOVA reveals significant main effects of treatment ($F_{1,45} = 16.93$, $P < 0.01$) and condition ($F_{2,45} = 5.40$, $P < 0.01$), as well as a significant treatment × condition interaction ($F_{2,45} = 4.25$, $P < 0.05$). Vehicle injection in CPIP rats did not produce significant nociceptive behaviours at 2 or 7 days post-reperfusion as compared to vehicle injections in sham control rats. L-NIO-induced significant nociceptive behaviour at 2 days post-reperfusion as compared to vehicle injections in CPIP rats ($P < 0.01$), but not at 7 days post-reperfusion as compared to vehicle-injected CPIP rats. L-NIO also did not produce greater nociceptive behaviours in sham controls, as compared to vehicle injection.

4. Discussion

Our main finding here is that CPIP rats with mechanical allodynia display both exaggerated NE-evoked nociceptive behaviours and enhanced vasoconstrictive responses to NE. CPIP responders displayed pain responses to intradermal NE, while sham rats did not. Our results parallel those found in CRPS patients where intradermal NE evokes intense abnormal pain lasting tens of minutes [2,58]. CPIP responders also show exaggerated decreases in blood flow in response to NE. CRPS patients also have increased vasoconstrictive responses to NE [3,8,55]. Although early in the disease CRPS patients have reduced sympathetic function [9,50,51,63,64], later in the disease CRPS patients have greater decreases in blood flow in response to sympathetic stimulation [1,5,24,64], and exhibit denervation supersensitivity of vasoconstrictive adrenoceptors [30].

As well as exhibiting alterations relative to sham treatment after close arterial NE injections, the ipsilateral CPIP hind paw also showed vasoconstrictive hypersensitivity as compared to the contralateral paw following intravenous NE administration. Thus, while our relatively high doses of NE may have general systemic effects following close arterial administration, the vasoconstrictive hypersensitivity clearly depends on local alterations in the CPIP hind paw.

CPIP non-responders did not exhibit enhanced nociceptive responses to NE on day 2, but did on day 7. Thus, CPIP non-responders may have borderline pathology that worsens with time. Importantly, CPIP non-responders also did not exhibit significantly enhanced vasoconstrictive responses to NE. These findings strengthen the relationship between enhanced NE-induced nociceptive and vasoconstrictive responses in CPIP rats, by showing that rats with allodynia exhibit both painful and vascular NE hypersensitivity, while those without allodynia do not. The relationship is not absolute, since 7-day CPIP non-responders exhibited nociceptive responses to NE, but failed to exhibit significantly greater NE-induced vasoconstriction. Of course, it is impossible to directly compare these phenomena, since we used different routes of administration, and the rats were conscious during behavioural experiments and anesthetised during laser Doppler experiments.

The relationship between NE-induced exaggerated nociception and enhanced vasoconstriction is further supported by the finding that both were significantly reduced by α_1- and α_2-adrenergic receptor antagonists. Mechanical allodynia in CPIP rats is also reduced by a non-selective adrenergic receptor antagonist, phentol-

amine, and an α_1-adrenergic receptor antagonist, prazosin [66]. While no clinical studies have examined whether α-adrenergic antagonists alleviate exaggerated NE-induced pain in CRPS patients, the α_1-adrenergic agonist, phenylephrine, produces painful responses similar to NE [17,36a]. In contrast, α_2-adrenergic antagonists, such as phentolamine [4,45] and phenoxybenzamine [25,41], are used to treat pain and allodynia in CRPS patients, and to diagnose SMP.

We also demonstrated that co-administration of an NO donor (sodium nitroprusside) results in a dose-dependent attenuation of NE-evoked nociception. SNP has strong vasodilator activity that occurs following the release of NO. We hypothesize that the NO-mediated vasodilatation counteracts the vasoconstrictive effect of NE, as has previously been documented [56]. SNP has also been shown to reduce thermal hyperalgesia induced by NE in capsaicin-treated skin in human subjects [22], and we have shown that systemic SNP reduces mechanical allodynia in CPIP rats [65]. Administering another NO donor, SIN-1, at doses that are anti-allodynic, also attenuates NE-evoked nociception. NO-based vasodilators have been used to alleviate CRPS pain [27,37].

To further examine the relationship between vasoconstriction and NE-induced nociception, we tested the nociceptive effects of hind paw injection of a non-adrenergic vasoconstrictive agent, vasopressin, and an eNOS inhibitor, L-NIO. We found that both were able to induce nociception in CPIP rats. Although an analgesic role for systemically and spinally administered vasopressin is known [6,57], the effects of vasopressin on nociceptors have not been studied. However, after spinal nerve ligation, vasopressin evokes an excitation of axotomized afferents similar to that evoked by NE, suggesting vasoconstriction may be relevant to the effects of NE [26]. Also, both NE and vasopressin produce thermal hyperalgesia in capsaicin-treated skin in human subjects at doses that reduce blood flow [21].

L-NIO, by inhibiting the synthesis of NO, reduces NO-induced vasodilatation. Although it produces no significant nociceptive behaviours in sham rats, L-NIO produces nociceptive behaviours in CPIP rats similar to those produced by NE and vasopressin. Thus, nociceptive behaviours are induced in CPIP rats either by enhancing vasoconstriction with adrenergic (NE) or non-adrenergic (vasopressin) vasoconstrictors, or by reducing NO-mediated vasodilatation with an eNOS inhibitor (L-NIO). It would be interesting to investigate parallels between vascular and nociceptive effects of vasopressin and NO in a future study. Although there is a large literature indicating that eNOS and NO are critical players in vasoregulation, and that NO is a potent vasodilator [40], the role of eNOS and vascular NO in CRPS has received little attention. NOS is usually thought to be pro-nociceptive spinally [54], and intrathecal as well as systemic eNOS inhibitors attenuate pain in various pain models [20,42,44]. In neuropathic pain, NO is often thought to play a pro-nociceptive role by generating nitrogen free radicals and causing vasodilatation that facilitates inflammatory processes [28,35,36]. We hypothesize that in SMP, chronic tissue ischemia and vasoconstrictor hypersensitivity are over-riding factors that allow the major vasodilator role of endothelial NO to be beneficial rather than pro-nociceptive. Importantly, NO synthase activity and NO-dependent vasodilatation have been shown to be compromised after I–R injury [62], perhaps explaining why endogenous NO may not compensate for vasoconstrictor hypersensitivity in CPIP rats or CRPS patients.

In CRPS it has been suggested there is direct sympathetic–sensory coupling that underlies *de novo* adrenergic sensitivity in primary afferent neurons. Nerve injury induces sprouting of sympathetic fibers around the cell bodies of DRG neurons [14,38] and in the skin [68], as well as an upregulation of α-adrenoceptors in DRG neurons [7,13,67]. An upregulation of adrenergic receptors has been documented in the skin of CRPS-I patients [23], although it has not been determined whether the receptors are on nociceptors. After spinal nerve ligation the number of axotomized L5 afferent nerves that respond to NE or sympathetic stimulation is dramatically increased following sustained vasoconstriction of the DRG, and the DRGs exhibit abnormal neuronal responses to the vasoconstrictors NE, angiotensin and vasopressin [26], suggesting that sympathetic–sensory coupling is indirect and depends on intervening vasoconstriction.

Vasoconstriction-dependent *indirect* sympathetic–sensory coupling implies that vascular adrenergic hyper-responsiveness may be an alternative cause of adrenergic sensitivity of primary afferents in SMP. While not well studied in animal models of SMP, vascular adrenergic hyper-responsiveness has been demonstrated in various animal models of I–R injury. I–R injury of the rat tail [52] and cremaster muscle [34] results in enhanced vasoconstrictor responses to adrenergic stimulation. In humans, chronic limb ischemia associated with peripheral arterial disease has been shown to result in enhanced responsiveness of α-adrenergic receptors [29]. Indirect sympathetic–sensory coupling may depend more on upregulation of adrenoceptors on vascular smooth muscle cells than on primary afferents. Evidence shows there is an upregulation of both α_1- and α_2-adrenoceptors in vascular smooth muscle cells after I–R injury of the rat hind limb [48]. The vascular α-adrenoceptor upregulation occurs within 1 h following reperfusion, much earlier than the onset of 7–14 days observed for upregulation in DRG cells after spinal nerve ligation or partial sciatic nerve transection [7,13]. This is important since we have observed mechanical allodynia as early as 8 h after

reperfusion and abnormal NE-induced nociception at 2 days following reperfusion in CPIP rats. Although vascular α-adrenoceptor upregulation may contribute to the pathology in CPIP rats, it may be that more effective G-protein coupling of adrenergic receptors [52], or reduced NE clearance after ischemia, may also be important factors in the NE hypersensitivity of CPIP animals.

5. Conclusions

We have shown here that exaggerated NE-induced nociception in CPIP rats is paralleled by enhanced vasoconstriction in the hind paw, and that both are relieved by α-adrenergic antagonists. NE-induced nociception is also relieved by a vasodilator and mimicked by a non-adrenergic vasoconstrictor or an eNOS inhibitor. We hypothesize that CPIP rats, and at least some CRPS-I patients, have an upregulation of vascular adrenergic receptors. The pain of CPIP and CRPS-I may depend on chronic tissue ischemia that is dependent on, or exacerbated by, an indirect sympathetic–afferent coupling with an intervening role of enhanced α-adrenoceptor-mediated vasoconstriction.

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