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Chronic post-ischemia pain (CPIP): a novel animal model of complex regional pain syndrome-Type I (CRPS-I; reflex sympathetic dystrophy) produced by prolonged hindpaw ischemia and reperfusion in the rat

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Abstract

A neuropathic-like pain syndrome was produced in rats following prolonged hindpaw ischemia and reperfusion, creating an animal model of complex regional pain syndrome-Type I (CRPS-I; reflex sympathetic dystrophy) that we call chronic post-ischemia pain (CPIP). The method involves placing a tourniquet (a tight fitting O-ring) on one hindlimb of an anesthetized rat just proximal to the ankle joint for 3 h, and removing it to allow reperfusion prior to termination of the anesthesia. Rats exhibit hyperemia and edema/plasma extravasation of the ischemic hindpaw for a period of 2–4 h after reperfusion. Hyperalgesia to noxious mechanical stimulation (pin prick) and cold (acetone exposure), as well as mechanical allodynia to innocuous mechanical stimulation (von Frey hairs), are evident in the affected hindpaw as early as 8 h after reperfusion, and extend for at least 4 weeks in approximately 70% of the rats. The rats also exhibit spontaneous pain behaviors (hindpaw shaking, licking and favoring), and spread of hyperalgesia/allodynia to the uninjured contralateral hindpaw. Light-microscopic examination of the tibial nerve taken from the region just proximal to the tourniquet reveals no signs of nerve damage. Consistent with the hypothesis that the generation of free radicals may be partly responsible for CRPS-I and CPIP, two free radical scavengers, *N*-acetyl-L-cysteine (NAC) and 4-hydroxy-2,2,6,6-tetramethylpiperydine-1-oxyl (Tempol), were able to reduce signs of mechanical allodynia in this model.

Keywords: Complex regional pain syndrome-Type I; Chronic post-ischemia pain; Sympathetic dystrophy

Growing evidence suggests that complex region pain syndrome-Type I (CRPS-I, reflex sympathetic dystrophy) may depend in part on tissue ischemia. Skin capillary hemoglobin oxygenation (HbO_2) is lower (Koban et al., 2003), and skin lactate is increased, reflecting enhanced anaerobic glycolysis (Birklein et al., 2000a,b) in CRPS-I limbs. Also, cold CRPS-I limbs have impaired nutritive skin blood flow (Kurvers et al., 1995). Muscle tissue in amputated CRPS-I limbs was found to exhibit lipofuscin pigment, atrophic fibers, and severely thickened basal membrane layers of the capillaries, consistent with oxidative stress and ischemic conditions resulting from microangiopathy in muscle tissue (van der Laan et al., 1998b). There is also an impairment of

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high-energy phosphate metabolism in muscle tissue of CRSP-I limbs (Goris, 1998; Heerschap et al., 1993), suggestive of lowered mitochondrial oxygen supply.

Goris (1998) argued that CRPS-I depends on an exaggerated inflammatory response. Thus, there is increased density of perfused vessels, higher capillary filtration capacity (an index of microvascular permeability), and plasma extravasation in the affected limb in the early stages of CRPS-I (Matsumura et al., 1996; Oyen et al., 1993; Schurmann et al., 2001). While these changes are accompanied by high arterial blood flow, there is an elevated peripheral venous pressure, and arteriovenous shunting in the affected limb of CRPS-I patients (Matsumura et al., 1996; Schurmann et al., 2001). Thus, there is high arterial flow to the CRPS-I limb, but low oxygen consumption, as well as high lactate flux-indicative of tissue ischemia (Goris, 1991, 1998). CRPS-I may depend on ischemia–reperfusion (IR) injury which also produces arteriovenous shunting (Kennedy et al., 1981), and is known to contribute to ischemic contracture and compartment syndrome in traumatic (Hoover and Siefert, 2000) or tourniquet (Blaisdell, 2002) shock.

Previous studies in rats show that transient (5–12 min) tourniquet-induced tail IR causes hyperalgesia lasting at least 2 h (Gelgor et al., 1986; Vidulich and Mitchell, 2000). Prolonged (2 h) tourniquet-induced IR of the rat hindpaw produces an immediate hyperemia on reperfusion, and subsequent persistent hindpaw edema (Somogyi and Selye, 1969). We examine here whether prolonged (3 h) IR of the rat hindpaw produces inflammatory and pain symptoms similar to CRPS-I in humans.

Considerable evidence suggests that oxygen free radicals may contribute to IR injury and possibly also CRPS-I. Hindlimb IR increases various free radicals in postcapillary venules (Blaisdell, 2002; Yassin et al., 1997). Free radical scavengers reduce heat-hyperalgesia in rats with chronic constriction injury of the sciatic nerve (Khalil and Khodr, 2001; Khalil et al., 1999; Tal, 1996). CRPS-I symptoms are relieved following treatment with free radical scavengers (Geertzen et al., 1994; Goris, 1985, 1998; Goris et al., 1987; Perez et al., 2003; Zurrmond et al., 1996), and the incidence of CRPS-I after wrist fractures may be reduced by pre-emptive treatment with the anti-oxidant vitamin C (Amadio, 2000; Cazeneuve et al., 2002; De Lange-de Klerke, 2000; Zollinger et al., 1999). Thus, another purpose of this study was to examine the potential anti-hyperalgesic effects of free radical scavengers in our animal model of CRPS-I.

1. Materials and methods

1.1. Animals

The present studies employed male Long Evans hooded rats (275–325 g, Charles River, Quebec). Rats 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 (Zimmermann, 1983).

1.2. Hindpaw ischemia and reperfusion

Chronic post-ischemia pain (CPIP) was generated following exposure to prolonged hindpaw ischemia and reperfusion. Rats were anesthetized over a 3 h period with a bolus (40 mg/kg, i.p.) and chronic i.p. infusion of sodium pentobarbital for 2 h (13 mg/h for first hour, 6.5 mg/h for second hour). After induction of anesthesia, a Nitrile 70 Durometer O-ring (O-rings West, Seattle, WA) with 7/32 in. internal diameter was placed around the rat's left hindlimb just proximal to the ankle joint. The O-rings were selected to produce a tight-fit that produced ischemia similar to that produced by a blood pressure cuff inflated to 350 mmHg, and were left on the limb for 3 h. We standardized the position of the O-ring to a point on the limb just proximal to the medial malleolus. The application was standardized by sliding the O-ring off the outside of a 3 cm^3 syringe (that was cut in half), after the hindpaw was inserted into the syringe barrel as far as possible. 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 hindpaw.

1.3. Hyperemia and plasma extravasation

Hyperemia was examined by measuring the temperature of the plantar surface of the hindpaws using a thermocouple probe connected to a transducer (BAT-12, Physitemp, Clifton, NJ). A temperature measurement was based on an average of three replicate recordings taken at various time points between 5 min and 4 h after reperfusion. Measurements were obtained from separate groups of CPIP ($N=6$) and sham ($N=6$) rats, and a hyperemia score for each animal was generated by subtracting the temperature measurement of the contralateral hindpaw from that of the ipsilateral hindpaw.

Edema was assessed by determining the degree of plasma extravasation using a spectrophotometric analysis of Evans Blue dye extravasation from the ipsilateral, as compared to the contralateral, hindpaw (Yashpal and Coderre, 1998). Rats were briefly anesthetized with halothane (4%) and given an intravenous (tail vein) injection of Evans Blue dye (50 mg/kg in 2.5 ml/kg) 30 min prior to the desired measurement time. Thus, at 2, 12 or 24 h after reperfusion ($N=14$, 6, 16 for the three time points), or 2 h after sham treatment ($N=6$), rats were

re-anesthetized with sodium pentobarbital (100 mg/kg, i.p.), and received an intracardiac perfusion with 0.9% saline to flush blood from the circulation. The ipsilateral and contralateral hindpaws were then removed by amputation at the ankle joint. The hindpaws were next incubated in 4 ml of formamide at 70 °C for 24 h to extract Evans Blue dye from the tissue. After cooling to room temperature, the amount of extravasated dye from each hindpaw was determined by spectrophotometric measurement of absorbance at a wavelength of 655 nm and interpolation from a linear standard curve, and an edema score for each animal was generated by subtracting the amount of extravasated Evans blue dye of the contralateral hindpaw from that of the ipsilateral hindpaw.

1.4. Mechanical and thermal sensitivity

The plantar surface of the ipsilateral and contralateral hindpaws of CPIP ($N=15$) and sham ($N=10$) rats was tested for mechano-allodynia, mechano-hyperalgesia, cold-allodynia and heat-hyperalgesia (in that order) over a period between 8 h and 4 weeks after hindpaw IR. Measurements of mechano-allodynia preceded cold-allodynia in order to avoid cold-induced reductions in mechanical thresholds (Kauppila, 2000). Rats that did not exhibit positive sensory symptoms (i.e. hyperalgesia or allodynia) by the 48 h measurement were excluded from the data presented here; approximately 70% of rats showed positive sensory symptoms. Pilot experiments with Sprague–Dawley rats (Harlan Indianapolis breeding colony, Fredrick, MD) revealed a similar incidence, but a reduced duration of symptoms.

1.4.1. Hindpaw mechano-allodynia

Mechano-allodynia of the hindpaw was assessed by measuring the hindpaw withdrawal response to von Frey filament stimulation according to a modification of the method described by Chaplan et al. (1994). In brief, animals were placed in a Plexiglas® box ($21\times16\times27$ cm^3) with a wire grid bottom through which the von Frey filaments (Stoelting) were applied to the plantar surface of the hindpaw. 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 5 times; a response to three of the five applications was counted as positive. The minimum stimulus 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 using the convention, x = withdrawal, o = no withdrawal, and the 50% response threshold was interpolated using the formula: 50% g threshold $= (10^{[x+k\delta]}_{f})/10,000$, where x_f = value (in log units) of the final von Frey hair used; k = tabular value (see Chaplan et al., 1994) for pattern of positive/negative responses; and δ = mean difference (in log units) between stimuli (here 0.224). Hairs (nylon monofilaments; Stoelting, Woodale, IL) were from the standard Semmes–Weinstein series (Semmes et al., 1960).

1.4.2. Hindpaw mechano-hyperalgesia

Mechano-hyperalgesia of the hindpaw was assessed using a modification of the pin prick method described by Tal and Bennett (1994). With the rats standing on the wire mesh floor and confined beneath an inverted plastic box (described above), the point of a blunted 23 gauge needle was applied to the skin of the heel (touching, but not penetrating). Normal rats respond with a very small and brief withdrawal. CPIP rats, like neuropathic rats (e.g. CCI rats), respond most often with a withdrawal that is clearly exaggerated in amplitude and duration. Behavioral responses to the pin prick were rated according to the following scale: 0 = no response; 1 = rapid paw flicking, stamping, or shaking (less than 1 s); 2 = repeated paw stamping, shaking, or paw lift less than 3 s; 3 = above behaviors or hindpaw licking for more than 3 s; 4 = above behaviors for more than 3 s and hindpaw licking for more than 3 s. An additional point was added if any vocalizations occurred.

1.4.3. Hindpaw cold-allodynia

Cold-allodynia of the hindpaw was assessed using a modification of the acetone drop method described by Choi et al. (1994). With the rats standing on the wire mesh floor and confined beneath an inverted plastic cage, a drop of acetone was placed on the skin of the heel. Normal rats either ignore the stimulus or occasionally respond with a very small and brief withdrawal. CPIP rats, like CCI rats, respond most often with a withdrawal that is clearly exaggerated in amplitude and duration. Behavioral responses to the acetone drop were rated according to the same scale described above for the pin prick test.

1.4.4. Hindpaw heat-hyperalgesia

Heat-hyperalgesia of the hindpaw was tested using methods described by Hargreaves et al. (1988). Briefly, the rat was placed within a plastic compartment atop a glass floor; a light source beneath the floor was aimed at the skin of the fat part of the heel. The nocifensive withdrawal reflex interrupts the light reflected from the heel onto a photocell and automatically turns off the light and a timer. The intensity of the light was adjusted at the start of the experiment such that average baseline latencies were about 10 s and a cut-off latency of 20 s was imposed. Latency to withdrawal defines the heat–pain threshold. A latency score was based on the average of the two most consistent of three replicate recordings, which were obtained alternately from each hindpaw 5-min apart. Data were converted to percentage change from baseline since there was an approximately 1.75 s difference in the baseline latencies between the CPIP and sham rats for both ipsilateral and contralateral heat-hyperalgesia trials.

1.5. Histology

The tibial nerves of six animals were examined: four at 48 h and two at 7 days after IR. When deeply anesthetized following a sodium pentobarbital overdose (100 mg/kg, i.p.), the rats were perfused transcardially with 150 ml of a phosphate buffered solution containing 0.1% sodium nitrate, followed by 200 ml of freshly prepared 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer. Segments of the tibial nerve from the ankle (just proximal to the location, where the tourniquet had been applied) were harvested bilaterally and post-fixed in the same solution. Following incubation in 10% sucrose, the nerves were embedded in epoxy (Epon), sectioned at 1 μm, and stained with toluidine blue.

1.6. Free radical scavenger trial

Rats were administrated the agents 4-hydroxy-2,2,6,6-tetramethylpiperydine-1-oxyl (Tempol) or *N*-acetylcysteine (NAC) to establish the potential anti-allodynic effects of free radical scavengers. Tempol is a nitroxide free radical scavenger (Thiemermann, 2003); NAC is a precursor of glutathione, an endogenous anti-oxidant (Skrzydlewska and Farbiszewski, 1999). After a baseline von Frey trial, rats received a 3 h tourniquet (O-ring) exposure and reperfusion as described above. Rats were then tested for von Frey thresholds at 48 h after reperfusion, both before and 30 min after treatment with either Tempol (250 mg/kg, i.p.) or NAC (500 mg/kg, i.p.) doses that produce effective anti-oxidant effects in vivo (Cuzzocrea et al., 2001; Sener et al., 2003).

2. Results

As compared to a normal hindpaw (Fig. 1A), a hindpaw with an O-ring tourniquet shows clear evidence of hypoxia, becoming cold and cyanotic (Fig. 1B). Towards the end of the 3 h tourniquet application, some rats developed a moderate degree of fluid accumulation in subcutaneous tissue on the dorsal surface on the hindpaw, although the hindpaw remained cyanotic (not shown). Immediately following reperfusion, there is a period of hyperemia and vasodilatation, in which the hindpaw becomes warm, engorged with blood and highly edematous-remaining so for a period of 3–4 h (Fig. 1C). By 24 h after reperfusion, the hyperemia and edema subsides, and the hindpaw takes on a dry and shiny appearance (Fig. 1D).

2.1. Hyperemia and plasma extravasation

Hindpaw temperature recordings were taken between 5 min and 4 h after reperfusion, and measurements were expressed as temperature difference between the ipsilateral and contralateral hindpaws for each rat. Two way repeated ANOVA revealed significant main effects of treatment

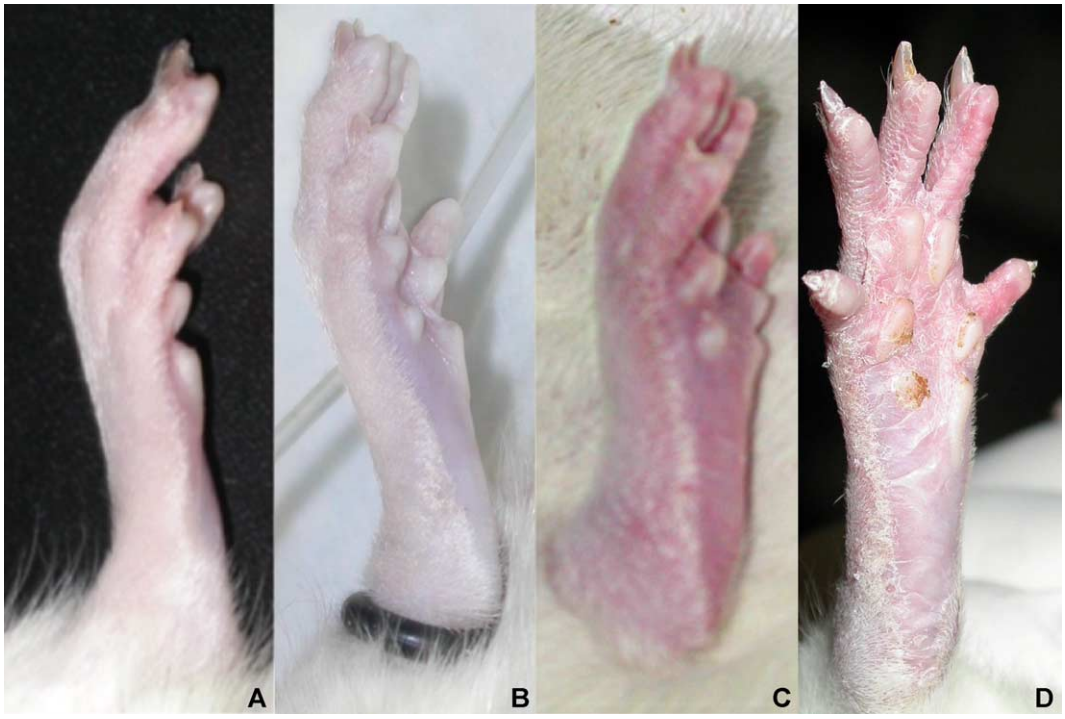

Fig. 1. Representative photographs of rat hindpaws take before tourniquet exposure (A), during tourniquet exposure (B), 5 min following reperfusion (C), and 24 h following perfusion (D). During tourniquet exposure (B), the hindpaw is cold and cyanotic, reflecting tissue hypoxia. Shortly after reperfusion (C) the hindpaw is hot, engorged with blood and edematous, reflecting an intense reactive hyperemia. At 24 h post-reperfusion (D), the hyperemia and edema subside, and the hindpaw appears dry and shiny.

group ($F(1,11)=6.06$, $P<0.05$) and time ($F(4,40)=5.05$, $P<0.01$), as well as a significant group×time interaction ($F(4,40)=7.14$, $P<0.001$). Compared to sham rats for which hindpaw temperature did not vary significantly over the 4 h of testing, the mean temperature difference between the ipsilateral and contralateral hindpaws of CPIP rats was significantly elevated above baseline between 5 min and 2 h after reperfusion ($P<0.05$, Dunnett's). The temperature difference peaked at 5 min following reperfusion, and returned to baseline levels by 4 h after reperfusion (Fig. 2A). In patients, a side-to-side temperature difference of 1 °C or greater is considered abnormal (Uematsu et al., 1988).

Hindpaw plasma extravasation assessments of CPIP rats were taken between 2, 12 and 24 h after reperfusion, and measurements were expressed as the difference in amount of extravasated blue dye between the ipsilateral and contralateral hindpaws for each rat. Compared to sham rats, which did not exhibit a significant difference between the ipsilateral and contralateral hindpaws at the 2 h time point, the mean difference in extravasated Evans Blue dye between the ipsilateral and contralateral hindpaws of CPIP rats was significantly elevated at 2 h after reperfusion ($F(3,38)=4.29$, $P<0.05$), and returned to normal by 12 h after reperfusion (Fig. 2B).

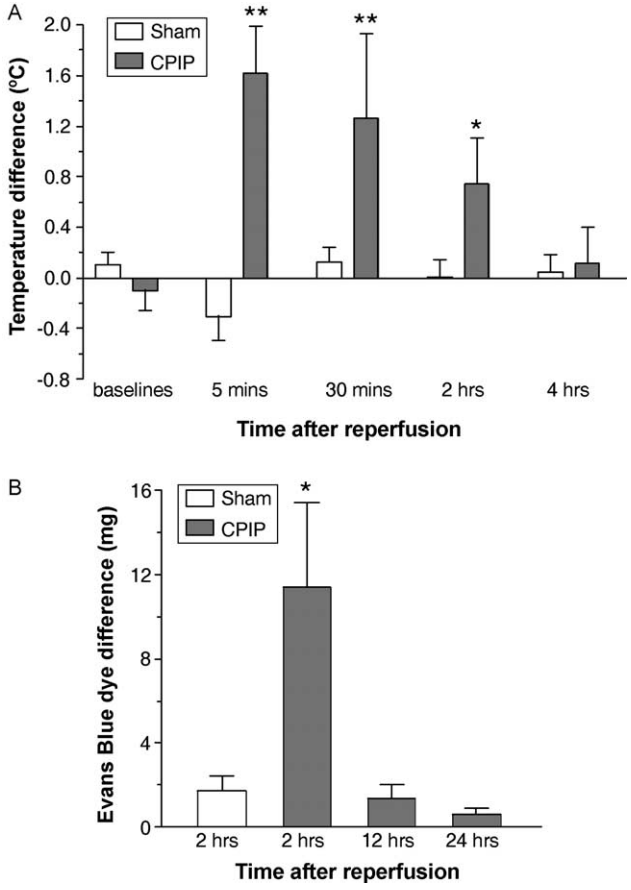

Fig. 2. Time course of the hyperemia and edema in CPIP and sham rats, as measured as the temperature difference (A) and the difference in extravasated Evans Blue dye (B) between the ipsilateral and the contralateral hindpaws of CPIP and sham rats. A significant increase in the ipsilateral–contralateral hindpaw temperature difference for CPIP rats, but not shams, was observed between 5 min and 2 h after reperfusion (A). Compared to the measurement for shams at 2 h, there was also a significant increase in the difference of extravasated Evans Blue dye (ipsilateral–contralateral) in CPIP rats at 2 h (B) (*$P<0.05$, **$P<0.01$).

2.2. Mechanical and thermal sensitivity

2.2.1. Hindpaw mechano-allodynia

von Frey thresholds for both the ipsilateral ($\chi^2(7)=1.04$, $P>0.05$) and contralateral ($\chi^2(7)=0.78$, $P>0.05$; Friedman ANOVA) hindpaws of sham rats did not vary across the 4 weeks of testing. In contrast, CPIP rats developed mechano-allodynia over a prolonged period in both the ipsilateral ($\chi^2(7)=31.6$, $P<0.001$) and contralateral ($\chi^2(7)=17.0$, $P<0.05$) hindpaw, with more pronounced effects on the ipsilateral side. Ipsilateral mechano-allodynia was present within 8 h following reperfusion, peaked at 4 days, and persisted for at least 4 weeks after reperfusion (Fig. 3A). Contralateral mechano-allodynia was also present within 8 h following reperfusion, peaked at 2 days, and persisted for 2 weeks after reperfusion (Fig. 3B).

2.2.2. Hindpaw mechano-hyperalgesia

Nociceptive responses to pin prick for both the ipsilateral ($\chi^2(7)=4.49$, $P>0.05$) and contralateral ($\chi^2(7)=5.59$, $P>0.05$) hindpaws of sham rats did not vary across the 4 weeks of testing. In contrast, CPIP rats developed mechano-hyperalgesia over a prolonged period in both the ipsilateral ($\chi^2(7)=28.0$, $P<0.001$) and contralateral ($\chi^2(7)=21.7$, $P<0.01$) hindpaw, with more pronounced effects on the ipsilateral side. Ipsilateral mechano-hyperalgesia peaked at 8 h, and persisted for at least 4 weeks after reperfusion (Fig. 4A). Contralateral mechano-hyperalgesia was more sporadic, but was evident at 8 h, 4 days, and 2 and 4 weeks after reperfusion (Fig. 4B).

2.2.3. Hindpaw cold-allodynia

Responses to cold for both the ipsilateral ($\chi^2(7)=1.26$, $P>0.05$) and contralateral ($\chi^2(7)=2.50$, $P>0.05$) hindpaws of sham rats did not vary across the 4 weeks of testing. In contrast, CPIP rats developed cold-allodynia over a prolonged period in both the ipsilateral ($\chi^2(7)=16.9$, $P<0.05$) and contralateral ($\chi^2(7)=16.9$, $P<0.05$) hindpaw, with more pronounced effects on the ipsilateral side. Ipsilateral cold-allodynia was evident at 8 h, peaked at 2 weeks, and persisted for at least 4 weeks after reperfusion (Fig. 5A). Contralateral cold-allodynia was again more sporadic, but was evident at 8 h, 4 days, and 2 and 4 weeks after reperfusion (Fig. 5B).

2.2.4. Hindpaw heat-hyperalgesia

The percentage change in nociceptive withdrawal latencies to noxious heat for both the ipsilateral and contralateral hindpaws of both sham and CPIP rats did not

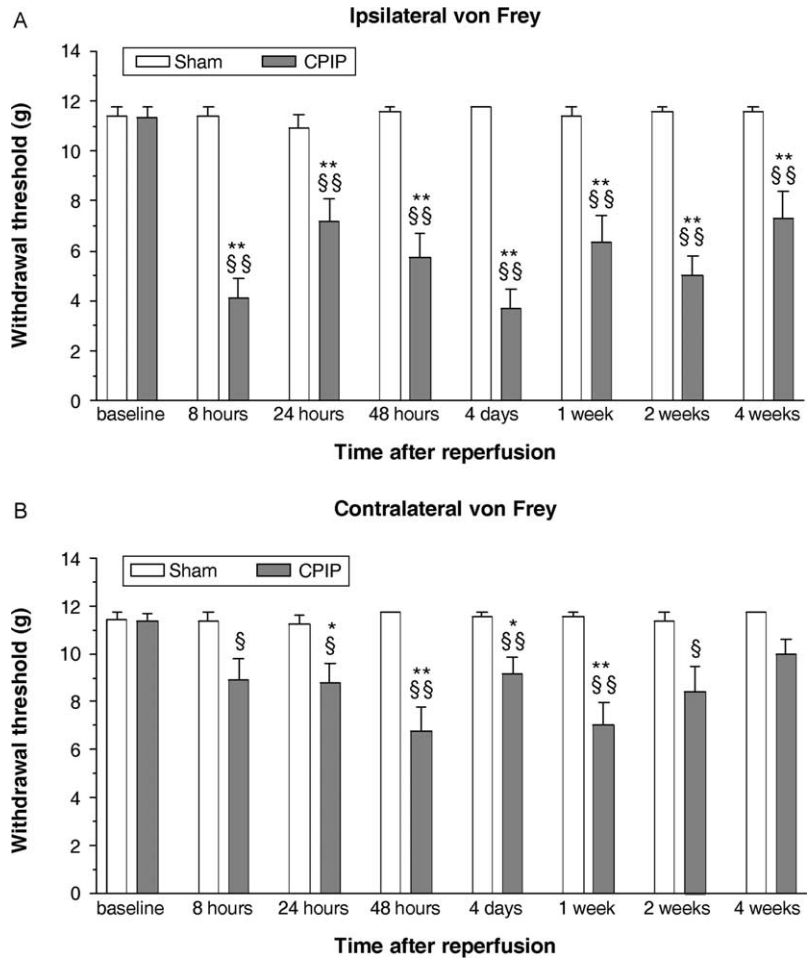

Fig. 3. Time course of mechano-allodynia in the ipsilateral (A) and contralateral (B) hindpaws of CPIP and sham rats, as determined by the von Frey test. Ipsilateral and contralateral withdrawal thresholds of sham rats did not change throughout the 4 weeks of testing. Withdrawal thresholds of CPIP rats were significantly reduced between 8 h and 4 weeks after reperfusion, ipsilaterally, and between 8 h and 2 weeks after reperfusion, contralaterally ($^{*}P<0.05$, $^{**}P<0.01$ from sham; $^{\S}P<0.05$, $^{\S\S}P<0.01$ from baseline).

vary significantly across the 4 weeks of testing. Thus, two-way repeated ANOVA revealed non-significant main effects of treatment group ($F(1,23)=0.23$, $P>0.05$) and time ($F(6,138)=0.69$, $P>0.05$), as well as a non-significant interaction of group×time ($F(6,138)=0.48$, $P>0.05$) for the ipsilateral latencies, and a non-significant main effects of group ($F(1,23)=1.02$, $P>0.05$) and time ($F(6,138)=0.82$, $P>0.05$), as well as a non-significant interaction of group×time ($F(6,288)=0.49$, $P>0.05$) for the contralateral latencies. In general, there was a trend for the withdrawal latencies of CPIP animals to *increase* (data not shown), although this trend did not reach statistical significance.

2.3. Histology

Light-microscopic examination found no evidence of degeneration in the ipsilateral tibial nerves of three of the four rats examined 48 h after IR, in either of the rats examined at 7 days after IR, or in any nerves taken from the contralateral side. The ipsilateral tibial nerve of one rat examined at 48 h after IR had about a dozen myelinated axonal profiles, scattered throughout the endoneurial compartment, that did not have a clearly delineated central core of axoplasm; we are uncertain as to whether these fibers were degenerating or whether their appearance was due to fixation or staining artifact.

2.4. Free radical scavenger trial

As demonstrated in the time course trials, 48 h following hindpaw IR both ipsilateral and contralateral von Frey thresholds were significantly lower than baseline (Fig. 6), indicative of mechano-allodynia in CPIP rats. Conversely, 30 min following treatment with either NAC (Fig. 6A) or Tempol (Fig. 6B), both ipsilateral and contralateral von Frey thresholds of CPIP rats were not different from baseline, suggesting that both NAC and Tempol reversed the mechano-allodynia in CPIP rats.

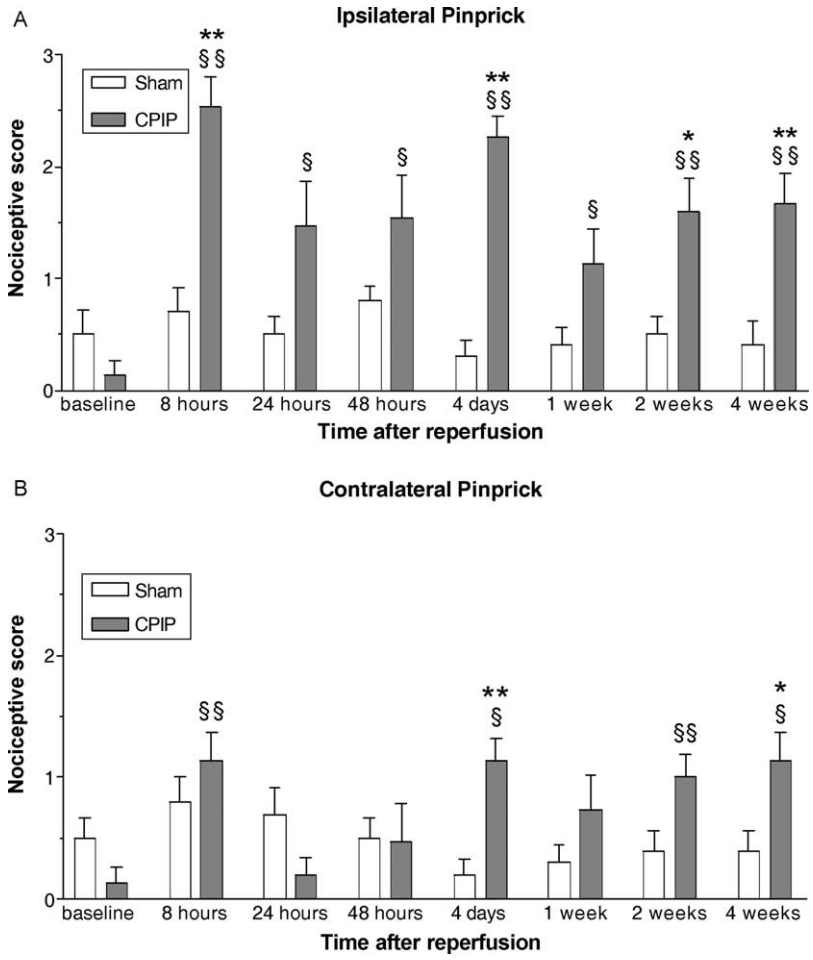

Fig. 4. Time course of mechano-hyperalgesia in the ipsilateral (A) and contralateral (B) hindpaws of CPIP and sham rats, as determined by the pin prick test. Ipsilateral and contralateral nociceptive scores of sham rats did not change throughout the 4 weeks of testing. Nociceptive scores of CPIP rats were significantly elevated between 8 h and 4 weeks after reperfusion, ipsilaterally, and at 8 h, 4 days and 2–4 weeks after reperfusion, contralaterally (*$P<0.05$, **$P<0.01$ from sham; $^{\S}P<0.05$, $^{\S\S}P<0.01$ from baseline).

3. Discussion

Rats exposed to prolonged hindpaw IR exhibited hyperemia and plasma extravasation in the ischemic hindpaw, acutely, and neuropathic pain-like symptoms, including hyperalgesia to noxious mechanical stimulation, cold-allodynia and mechano-allodynia, but not heat-hyperalgesia, in both the ischemic, and to a lesser extent, the contralateral hindpaw, chronically. However, there were no indications of nerve injury in CPIP rats. There was no loss or abnormality of motor function, no evidence of sensory anesthesia, and no microscopic evidence of nerve injury in the majority of the cases examined. Determination of the status of the unmyelinated C-fiber axons will require an electron microscopic study.

The development of CPIP as an animal model of CRPS-I is a physical injury model for this syndrome in which nerve injury is not evident. Other animal models of CRPS-I have been developed including that produced by sustained (10 min) tetanic stimulation of the sciatic nerve in the rat (Vatine et al., 1998, 2001), and that induced by continuous hindlimb intra-arterial infusion of a free radical donor, tert-butylhydroperoxide (tert-BuOOH) (van der Laan et al., 1997a,b, 1998a). An interesting parallel between the CPIP and the free radical donor model is the very significant appearance of bilateral symptoms. van der Laan et al. (1997b) found significant mechano-allodynia in the hindpaw contralateral to the tert-BuOOH infusion between 7 and 28 days after the infusion. Although contralateral effects have been described sporadically following various models of neuropathic and inflammatory pain (see Koltzenburg et al., 1999 for review), rarely are the effects so robust and long-lasting, as demonstrated in these two models. The robustness of these bilateral effects parallel findings of CRPS-I spreading to the contralateral side of some patients, reportedly as high as 16% (Allen et al., 1999; Maleki et al., 2000). We expect that the robust contralateral effects may depend on the higher degree of central sensitization that is obtained after injury of muscle tissue, as opposed to cutaneous tissue (Woolf and Wall, 1986).

The strength of the present CPIP model is that CRPS-I-like symptoms are induced by a physical injury that is

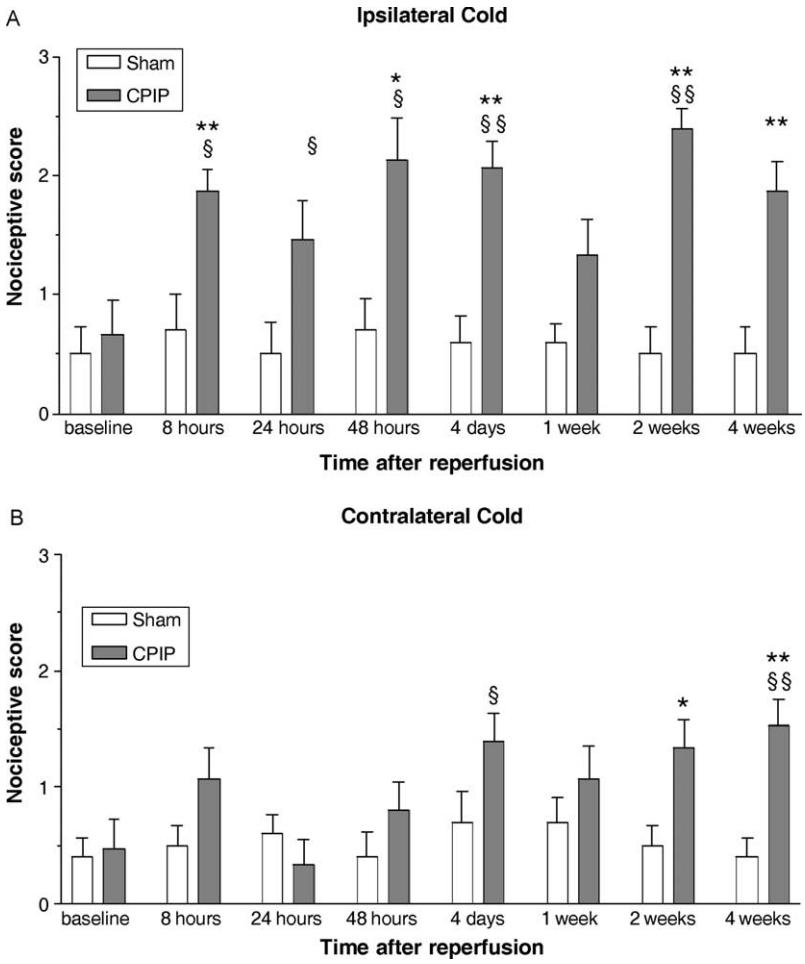

Fig. 5. Time course of cold-allodynia in the ipsilateral (A) and contralateral (B) hindpaws of CPIP and sham rats, as determined by the acetone drop test. Ipsilateral and contralateral nociceptive scores of sham rats did not change throughout the 4 weeks of testing. Nociceptive scores of CPIP rats were significantly elevated between 8 h and 4 days, and 2–4 weeks after reperfusion, ipsilaterally, and at 4 days and 2–4 weeks after reperfusion, contralaterally (*$P<0.05$, **$P<0.01$ from sham; $^{\S}P<0.05$, $^{\S\S}P<0.01$ from baseline).

comparable to those injuries seen in CRPS-I patients. CRPS-I commonly follows fractures, sprains, contusions and crush injuries, arthroscopic surgery, overly tight casting, and other edematous soft tissue injuries (Allen et al., 1999; Galer et al., 2000; Sandroni et al., 2003). A common feature of all these conditions is an early inflammatory response that has the potential to produce microvascular and ischemic changes in various tissues. Interestingly, the incidence of vascular complications after arthroscopic surgery (including reflex sympathetic dystrophy) are dramatically increased when a tourniquet time of 60 min or longer is used (Sherman et al., 1986). CRPS-I is not common after tourniquet application, chiefly because surgeons are aware of the dangers of prolonged tourniquet exposure. Typically tourniquets are not used longer than 2 h, and blood flow is intermittently re-established for the longer tourniquet times (Fletcher and Healy, 1983). Despite this, it has been established that after arthroscopic surgery there is typically more pain (Berga et al., 2002) and more dysfunction (Gutin et al., 1991) following tourniquet use.

We suggest that the symptomology that occurs after CPIP (early hyperemia and edema followed by long-lasting mechanical and cold-allodynia/hyperalgesia) resembles the two prominent phases of CRPS-I in humans (Birklein et al., 2000a,b; Wasner et al., 2001a,b). However, in patients hyperemia and oedema are not always present, and may be brief, or alternatively may be prolonged for many months or even years. There are even those who argue that CRPS-I limbs do not always progress from a hot edematous stage to a cold 'vasoconstrictive' stage (Bruehl et al., 2002). Nonetheless, the fact that IR injury leads to a persistent pain syndrome in rats, brings to light the possibility that similar mechanisms may contribute to some symptoms in CPIP rats and CRPS-I patients.

We expect that the extensive, but brief, ischemia in CPIP rats produces only an acute inflammatory response, while less extensive, but more prolonged, ischemia may result in a longer lasting inflammatory period in CRPS-I patients. Although the extent and course of the process may vary, the underlying mechanisms may be quite similar. The fact that CPIP rats do not exhibit a later cold limb or motor

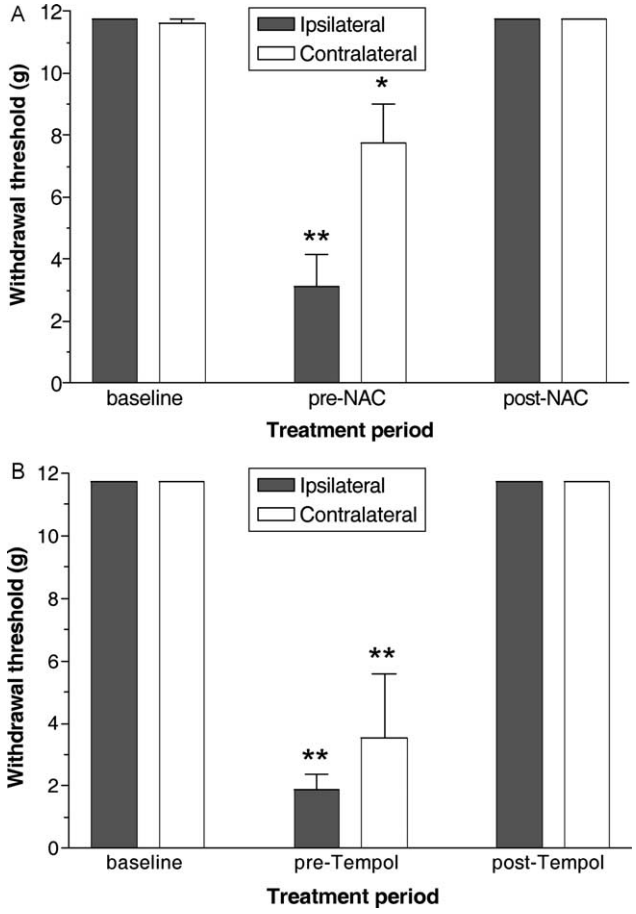

Fig. 6. Comparison of the mechanico-allodynia observed before and after post-treatment with NAC (A) or Tempol (B) 48 h after reperfusion in CPIP rats. Both NAC (500 mg/kg, i.p.) and Tempol (250 mg/kg, i.p.) reversed mechano-allodynia in the ipsilateral and contralateral hindpaws (*$P<0.05$, **$P<0.01$).

disturbances may reflect the relatively short period for which the animals were observed. However, prolonged or extensive tissue ischemia and reperfusion can produce an increasing myofascial dysfunction leading to motor disturbances (Hinik et al., 1997; Labbe et al., 1987). Hyperhidrosis that occurs in some CRPS-I patients does not appear to be evident in CPIP rats. However, rats have very few sweat glands, and they do not use sweating for temperature regulation. Thus, one would not expect to see sweating in association with vasodilatation, as occurs in humans (Janig and Habler, 2003). It is also true that as high as 50% of CRPS-I patients exhibit heat-hyperalgesia (Price et al., 1992; Tahmoush et al., 2000), while CPIP rats do not. Clearly, IR injury may not explain all symptoms experienced by all CRPS-I patients, which are arguably a fairly heterogeneous group. This heterogeneity is highlighted by other reports indicating that like for CPIP rats, heat-hyperalgesia is absent or infrequent in CRPS-I patients (see Guo et al., 2004).

The results here suggest we may be able to learn more about CRPS-I by studying the inflammatory processes that follow IR. Hindlimb IR produces various effects depending on the area and length of ischemia. When blood flow is occluded to the entire hindlimb, there is damage to muscle after 4 h and to peripheral nerves after 8 h of IR (Labbe et al., 1987; Steinau et al., 1988). Between 2 and 4 h of IR, there are microcirculatory changes (thrombosis, capillary endothelial cell swelling, leukocyte plugging) that result in increased vascular permeability to plasma proteins, interstitial edema (Harris et al., 1997; Strock and Manjo, 1969), and arteriovenous shunting (Kennedy et al., 1981). Before 4 h there is minor damage to muscle, but after 4 h the loss of nutrient blood flow causes muscle necrosis (Makitie and Teravainen, 1977). Ischemic conditions secondary to edema accumulation may lead to a compartment syndrome, where increased tissue pressure in an anatomical compartment compromises blood flow to muscles, nerves and bone causing tissue damage (Perry, 1988). Untreated compartment syndrome in muscle will lead to ischemic contracture—causing muscle stiffness and deformity (Hoover and Siefert, 2000). More extensive and/or longer ischemic periods lead to oxidative stress (lipid peroxidation) that damages the blood nerve barrier and causes endoneurial edema and nerve fiber degeneration (Saray et al., 1999).

We propose that CRPS-I, in some cases, depends on a microcirculatory abnormality that occurs following IR and persistent inflammation that occur after the initial insult. It may be significant that CRPS-I often follows fractures, sprains and arthroscopic surgery of knees and elbows (Allen et al., 1999; Galer et al., 2000). One can imagine that such injuries could be especially likely to produce compartment-like syndromes, where edema accumulates within muscle, joint capsules or other anatomical compartments, and leads to significant tissue ischemia. These events may lead to a persistent state of tissue ischemia, or borderline ischemia, which is likely to sensitize and activate the afferent innervation of the tissue. If true for muscle and/or periosteal nociceptors, this would cause a persistent, deep pain sensation. Activation of muscle or periosteal nociceptors, or skin C-fibers, may also lead to a central sensitization that would contribute to mechanical allodynia/hyperalgesia and cold-allodynia. Importantly, conditioning stimulation of muscle C-fibers produces greater central sensitization than does stimulation of cutaneous C-fibers (Wall and Woolf, 1984). Of note, strictly cutaneous injuries (e.g. burns and lacerations) are very infrequently cited as antecedents of CRPS-I.

Microcirculatory abnormalities may maintain CRPS-I symptoms. CRPS-I patients commonly report that exercise worsens their pain (Oyen et al., 1993), as would be expected if their muscles were borderline ischemic. Ischemia in bone and periosteal tissues might underlie the osteoporosis often found in CRPS-I patients (Kozin et al., 1976a,b; Mailis et al., 1994; Sudeck, 1902). Activity in sympathetic fibers of the affected limb would exacerbate any underlying ischemic condition, and this would explain the often important, but not necessarily causative, contribution of the sympathetic nervous system

to CRPS-I (Baron and Maier, 1996; Blumberg et al., 1997; Bonica, 1979; Wasner et al., 2001a,b).

Prolonged hindlimb IR has been shown to produce a well-documented cascade of inflammatory events, with a key role for reactive oxygen species (Blaisdell, 2002; Yassin et al., 1997). Hindlimb IR results in the production of the oxidants, superoxide, hydrogen peroxide, hydroxyl radical, perhydroxyl radical, singlet oxygen and peroxynitrite anion, initiated by the enzymes xanthine oxidase (Kellog, 1975; McCord, 1987) or NADPH oxidase (Inauen et al., 1989; Partrick et al., 1996). Xanthine oxidase and lipid peroxidase activity is increased in sciatic nerve of CCI rats (Khalil and Khodr, 2001; Khalil et al., 1999). Our investigations demonstrating that CPIP symptoms are reduced by post-treatment with free radical scavengers stresses the role of oxidants in the maintenance of neuropathic pain-like symptoms in this model of CRPS-I as well.

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