Topical combinations aimed at treating microvascular dysfunction reduce allodynia in rat models of CRPS-I and neuropathic pain

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Abstract

Growing evidence indicates that various chronic pain syndromes exhibit tissue abnormalities caused by microvasculature dysfunction in the blood vessels of skin, muscle or nerve. We tested whether topical combinations aimed at improving microvascular function would relieve allodynia in animal models of complex regional pain syndrome type I (CRPS-I) and neuropathic pain. We hypothesized that topical administration of either α_2 -adrenergic (α_{2A}) receptor agonists or nitric oxide (NO) donors combined with either phosphodiesterase (PDE) or phosphatidic acid (PA) inhibitors would effectively reduce allodynia in these animal models of chronic pain. Single topical agents produced significant dose-dependent anti-allodynic effects in rats with chronic post-

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Disclosures

JVR, AL and TJC are listed as inventors on a patent based on some of the results presented here and could potentially receive compensation from commercialization of the IP. All other authors have nothing to disclose.

ischemia pain, and the anti-allodynic dose-response curves of PDE and PA inhibitors were shifted 2.5–10 fold leftward when combined with non-analgesic doses of α_{2A} receptor agonists or NO donors. Topical combinations also produced significant anti-allodynic effects in rats with sciatic nerve injury, painful diabetic neuropathy and chemotherapy-induced painful neuropathy. These effects were shown to be produced by a local action, lasted up to 6 h after acute treatment, and did not produce tolerance over 15 days of chronic daily dosing. The present results support the hypothesis that allodynia in animal models of CRPS-I and neuropathic pain is effectively relieved by topical combinations of α_{2A} or NO donors with PDE or PA inhibitors. This suggests that topical treatments aimed at improving microvascular function may reduce allodynia in patients with CRPS-I and neuropathic pain.

Perspective—This article presents the synergistic anti-allodynic effects of combinations of α_{2A} or NO donors with PDE or PA inhibitors in animal models of CRPS-I and neuropathic pain. The data suggest effective clinical treatment of chronic neuropathic pain may be achieved by therapies that alleviate microvascular dysfunction in affected areas.

Keywords

Nociception; allodynia; analgesia; synergy; endothelial cell dysfunction

Introduction

Over the past several years, we have generated evidence that pain in an animal model of complex regional pain syndrome type I (CRPS-I) depends on local microvascular dysfunction^{14,56,93}. Evidence suggests that in rats with chronic post-ischemia pain (CPIP), and patients with CRPS, ischemic tissue injury leads to the generation of oxygen free radicals and pro-inflammatory cytokines which cause microvascular injury, including arterial vasospasm and capillary slow-flow/no-reflow in the blood vessels of muscle and nerve^{56,93}. Vasospasm (associated with reduced nitric oxide (NO) and increased vasoactive responses to norepinephrine (NE)), and capillary slow-flow/no-reflow (where blood flow to the many capillary beds is compromised), lead to poor muscle oxygenation and the build-up of muscle lactate, all of which contribute to the pain^{56,93}. Thus, CPIP animals have pain, allodynia, vasospasm, poor tissue perfusion, and oxidative stress^{14,56,93}. Evidence suggests that there is also microvascular dysfunction and poor tissue oxygenation in human CRPS limbs^{51,64,79,87}.

It has been known for many years that microvascular dysfunction and resulting oxidative stress contribute to the pain of angina^{57,85} and peripheral arterial disease^{8,69}. Recent parallel studies by other groups suggest that microvascular dysfunction and the resulting production of reactive oxygen and nitrogen species may contribute to neuropathic pain (including chronic constriction injury (CCI) of the sciatic nerve^{2,3,31}, painful diabetic neuropathy (PDN)^{21,24,42,45,48,74} and chemotherapy-induced painful neuropathy (CIPN)^{20,22,49,50}. Therefore, it is expected that treatments aimed at enhancing tissue oxygenation by reducing arterial vasospasm and capillary slow-flow/no-reflow will also relieve allodynia in animal models of nerve constriction, PDN and CIPN.

A predominant mediator controlling regional blood flow is the vasoconstrictive transmitter NE released from sympathetic post-ganglionic neurons, which contracts vascular smooth muscles^{28,39}. A second key mediator is the vasodilatory substance NO that is released from vascular endothelial cells and relaxes vascular smooth muscles^{72,89}. Thus, pharmacological agents that reduce NE release or binding, or agents that increase NO, are used to alleviate conditions with poor blood flow^{17,41}, including microvascular dysfunction. Various isoforms of phosphodiesterase enzymes (PDEs) also regulate blood flow⁹². Thus, PDE4,7 and 8 hydrolyse cAMP, PDE5,6 and 9 hydrolyse cGMP, and PDE1,2,3,10 and 11 hydrolyse both cAMP and cGMP⁷⁸. While PDE inhibitors that reduce cGMP hydrolysis enhance blood flow⁵⁹, PDE inhibitors that reduce cAMP hydrolysis also reduce platelet aggregation³⁸, decrease blood viscosity⁴⁴, and increase the flexibility of red blood cells⁴⁴, all of which relieve microvascular dysfunction. Thus, PDE inhibitors have been developed for conditions such as peripheral arterial disease (PAD)⁷⁷ and intermittent claudication⁴⁴, where microvascular injury leads to capillary slow flow/no reflow and reduced tissue oxygenation. Pentoxifylline is a PDE4 inhibitor commonly used to treat these ischemic disorders^{44,77}. A major metabolite of pentoxifylline, lisofylline shares many of the actions of its prodrug^{60,61,90}, but also inhibits the conversion of lysophosphatidic acid to phosphatidic acid (PA), a pathway critical to the production of various cytokines⁷⁶.

Although α_{2A} receptor agonists^{10,18,58} and NO donors^{1,33,63} have been used topically to alleviate chronic pain, PDE and PA inhibitors have not been previously used topically to alleviate pain. Furthermore, these agents have neither been combined systemically, nor combined in topical preparations for the treatment of chronic pain. However, α_{2A} receptor agonists, NO donors and PDE inhibitors have been used systemically in patients or preclinical animal models to treat pain associated with angina^{13,43,66}, PAD^{19,52,82}, CRPS^{18,40,63,74}, neuropathic pain^{32,53,60} and PDN^{16,24,48,84} indicating their usefulness in these syndromes. PA inhibitors have not been used to treat chronic pain, but should be equally or more effective than PDE inhibitors since they have anti-oxidant^{7,34}, anti-cytokine^{68,76}, anti-chemotactic^{70,91}, immunosuppressant^{15,23}, and mitochondrial protective¹² effects, in addition to the vasodilator⁶¹, anti-ischemic⁹⁰ and anti-platelet aggregation⁶² effects they share with PDE inhibitors.

In the present study, we have tested the hypothesis that agents aimed at increasing tissue oxygenation by increasing blood flow and reducing microvascular dysfunction (α_{2A} receptor agonists or NO donors, combined with PDE or PA inhibitors) can be used topically, either alone or in combination, on the hind paw to reduce allodynia in animal models of CRPS-I, nerve constriction, PDN and CIPN.

Materials and Methods

Animals

Male Long Evans or Sprague Dawley rats (225–250 g, Charles River, St. Constant, QC) arrived at least 7 days before experiments. Methods were approved by the animal care committee of McGill University, and conformed to ethical guidelines of the Canadian Council on Animal Care and the International Association for the Study of Pain.

Drugs

Drugs used included linsidomine (nitric oxide donor) (Tocris, Ellisville, MO), lisofylline (phosphatidic acid inhibitor) (Cayman Chemical, Ann Arbor, Michigan), apraclonidine (α_2 adrenergic receptor agonist), clonidine (α_2 adrenergic receptor agonist), pentoxifylline (phosphodiesterase inhibitor) and S-nitroso-N-acetyl- penicillamine (SNAP) (nitric oxide donor) (all obtained from Sigma-Aldrich, St. Louis, MO).

Drug formulations

Ointment-type formulations containing the above-mentioned drugs were formulated using a composite, water-soluble polyethylene glycol base system consisting of carbowax (PEG 3350) and PEG 400 (both from Sigma-Aldrich, St. Louis, MO) in the ratio of 60:40 respectively. All topical formulations were water-miscible/soluble ointments used to reduce irritation at the point of application and to ensure uniform and fast absorption. In the experiments with CIPN rats, 0.5% low molecular weight dextran sulfate was added as a tissue penetration enhancer. Briefly, the required amounts of the active ingredients were first weighed out and then added to the already molten base in decreasing order of their melting points, and stirred well. After uniform melting, the formulation was brought to room temperature to ensure proper solidification. A standard amount of 150 mg (mean $\pm$ SEM = 150.88 ± 2.7 mg) of the ointment was rubbed into the skin of the plantar and dorsal rat hind paw for 15 seconds using gloved finger tips, and the animals were immediately transferred to their respective cages. No rats were ever observed licking their hind paws after ointment application.

Chronic post-ischemia pain (CPIP)

Chronic post-ischemia pain (CPIP) was generated following prolonged hind paw ischemia and reperfusion, as previously described¹⁴. Briefly, male Long Evans rats were anesthetized over a 3 h period with a bolus (55 mg/kg, i.p.) followed by chronic i.p. infusion (0.15 ml/h) of sodium pentobarbital (Ceva Santé Animale, Libourne, France) for 2 h. After induction of anesthesia, a Nitrite 70 Durometer O-ring (O-rings West, Seattle, WA) with 5.5 mm internal diameter was placed around the rat's left hind limb proximal to the ankle joint. The tight-fitting O-ring produces a complete blockade of arterial blood flow as measured using laser Doppler flowmetry⁵⁶. The ring was then left in place for 3 h, and the rats recovered from anesthesia 30–60 min following reperfusion. Rats were tested between 2 and 14 days post-IR injury.

Chronic constriction injury (CCI) of the sciatic nerve

Unilateral mononeuropathy was produced in rats using CCI of the sciatic nerve, as described by Bennett and Xie⁶. Briefly, male Long Evans rats were anesthetized with sodium pentobarbital (55 mg/kg, i.p.), the common left sciatic nerve was exposed by skin incision and blunt dissection just above the trifurcation point, and four loose ligatures were then made with 4-0 chromic gut suture around the nerve, with about 1 mm spacing in between. The wound was then closed in layers with 3-0 silk thread and wound clips. The animals were then transferred to their home cages and left to recover. Rats were tested between 7 and 14 days post-CCI.

Diabetic neuropathy

Diabetic neuropathy was induced in male Sprague Dawley rats by a single injection of streptozotocin (STZ, 60 mg/kg, i.p.), and the rats were given 10% sucrose solution in their drinking water for 48 h after the injection. Blood samples from the tail vein were taken at 72 h and at one and two weeks following STZ injection, and only rats with blood glucose levels above 300 mg/dl (i.e., diabetic rats) were used. Rats were tested between days 14 and 21 after STZ injection.

Chemotherapy-induced painful neuropathy

Following habituation to the behavioural testing environment, male Sprague–Dawley rats (250–300 g) were injected intraperitoneally with the chemotherapeutic agent paclitaxel (Taxol®, Bristol–Myers–Squibb). Paclitaxel (2 mg/kg), prepared as a solution of 6 mg/ml in Cremophor EL vehicle diluted with saline was injected i.p. on four alternate days (days 0, 2, 4 and 6) as previously described⁸⁷. Rats were tested daily between days 28 and 43 post-paclitaxel treatment.

Mechanical sensitivity testing

The plantar surface of the ipsilateral hind paw was tested for mechanical allodynia (paw-withdrawal thresholds, PWTs) in CPIP and CCI rats, as well as bilaterally in PDN rats. Nylon monofilaments (von Frey hairs) were applied in either ascending (after negative response) or descending (after positive response) force as necessary to determine the filament closest to the threshold of response. Each filament was applied for 10 s or until a flexion reflex occurred. The minimum stimulus intensity was 1 g and the maximum was 15 g. Based on the response pattern, and the force of the final filament (5th stimulus after first direction change), the 50% threshold (grams) was calculated as $(10^{[X_f + k\delta]})/10000$ where X_f = filament number of the final von Frey hair used, k = value for the pattern of positive/negative responses and δ = mean difference in log unit between stimuli (here, $\delta = 0.220$, for more details see Chaplan et al.¹¹). All behavioural testing was performed with the experimenter unaware of the treatment condition.

Pharmacological treatments

Chronic post-ischemia pain—The topical formulations containing an NO donor, α_{2A} receptor agonist or a PDE or PA inhibitor were tested for their anti-allodynic effects, either singly, or in combination with each other, at concentrations selected from the published literature for use as topical agents. Accordingly, the rats received 150 mg of the respective ointment with the first half on the plantar aspect of their hind paws followed by the second half applied on the dorsal surface; in both cases by uniform gentle application using gloved fingers. The rats were monitored for several minutes after application to make sure they did not lick their paws. In the single drug pharmacological trials, clonidine was tested at 0.007, 0.015, 0.03 and 0.06% W/W ($n = 6$); apraclonidine at 0.005, 0.01, 0.02 and 0.04% W/W ($n = 6$); linsidomine at 0.2, 0.4, 0.8 and 1.6% W/W ($n = 8$); SNAP at 0.063, 0.125, 0.25 and 0.5% W/W ($n = 7$); pentoxifylline at 0.6, 1.2, 2.5 and 5% W/W ($n = 6$); and lisofylline at 0.063, 0.09, 0.125 and 0.25% W/W ($n = 10$).

In separate groups of CPIP rats, formulations containing pentoxifylline or lisofylline were tested in combinations with non-effective concentrations of clonidine, linsidomine, or SNAP. Accordingly, the formulations tested in the combination trials included clonidine (0.0075% W/W) + pentoxifylline (0.3, 0.6 and 1.2% W/W) (n = 9), linsidomine (0.4% W/W) + lisofylline (0.0313, 0.0625 and 0.0932% W/W) (n = 10), and SNAP (0.0625% W/W) + lisofylline (0.008, 0.015, 0.033 and 0.063% W/W) (n = 7). A third cohort of rats was used to confirm the local action of the tested formulations. For this study, the formulations were applied to the contralateral paw and the ipsilateral paw was tested for anti-allodynic effects. The most effective drug combinations were tested in this manner. Accordingly, the combinations included clonidine (0.0075% W/W) + pentoxifylline (0.6% W/W) (n = 9), linsidomine (0.4% W/W) + lisofylline (0.09% W/W) (n = 10) and SNAP (0.0625% W/W) + lisofylline (0.0625% W/W) (n = 7). In addition, vehicle (ointment base) application to the ipsilateral paw was also evaluated. All of the rats underwent initial baseline PWT assessment before application of the ointment followed by testing at 45 min post-application.

Nerve Constriction—In CCI rats, effective drug combinations (previously determined from the CPIP experiments described above) or vehicle ointment were tested for their anti-allodynic effects following either ipsilateral or contralateral hind paw application. The combinations included clonidine (0.0075% W/W) + pentoxifylline (0.6% W/W) (n = 7), apraclonidine (0.005% W/W) + lisofylline (0.03125% W/W) (n = 7), linsidomine (0.4% W/W) + pentoxifylline (0.3% W/W) (n = 7) and SNAP (0.0625% W/W) + lisofylline (0.0625% W/W) (n = 7). All of the rats underwent initial baseline PWT assessment before application of the ointment followed by testing at 45 min post-application.

Painful diabetic neuropathy—Three drug combinations were tested in PDN rats and age-matched control rats, including apraclonidine (0.0075% W/W) + pentoxifylline (0.6% W/W) (n = 4); apraclonidine (0.005% W/W) + lisofylline (0.0315% W/W) (n = 5); or linsidomine (0.4% W/W) + lisofylline (0.075% W/W) (n = 5) (separate groups). All of the rats underwent an initial pre-drug PWT assessment of both hind paws before application of the ointment to one hind paw followed by mechanical allodynia testing of both hind paws from 20–90 min post-application.

Chemotherapy-induced painful neuropathy—Two drug combinations were tested in CIPN rats, including apraclonidine (0.005% W/W) + lisofylline (0.03% W/W) (n = 10), or linsidomine (0.4% W/W) + pentoxifylline (0.4% W/W) (n = 10) (separate groups). All of the rats underwent an initial pre-drug PWT assessment of both hind paws before application of the topical combination to one hind paw and vehicle ointment base to the other hind paw daily for 15 days, followed by mechanical allodynia testing of both hind paws 1, 3, 6 and 24 h post-application on day 1 of treatment, or 3 h post-application on days 4, 8, 11 and 15 of treatment.

Data analysis

PWTs were averaged by group and treatment time and subjected to analysis of variance (ANOVA) using repeated measures. Pair-wise comparisons of group means were performed using Fisher's LSD tests after the observation of significant effects of drug treatment.

Shifts in drug anti-allodynic potency obtained by the use of combination treatments were illustrated by first calculating the difference between pre- and post-drug measures for each rat, then averaging these differences by treatment group. Mean differences were then plotted on a semilog scale of the amount of drug used per application. For each drug condition tested, unweighted linear regressions of PWT versus log dose were calculated for individual subjects, and the regression x-intercept estimates were then compared between drug alone and drug combination groups by one-way ANOVA in order to assess shifts in the dose-response profiles.

Results

Effects of topical treatments on mechanical allodynia in CPIP rats

In the single drug trials, hind paw topical application produced significant, dose-related anti-allodynic effects in CPIP rats for each of the six agents tested (Fig. 1A–F). Clonidine significantly elevated PWTs above pre-drug levels at 0.03 and 0.06% W/W ($P = 0.0104$ and $P = 0.0451$, respectively); apraclonidine at 0.02 and 0.04% W/W ($P = 0.0175$ and $P = 0.0008$, respectively); linsidomine at 0.8 and 1.6% W/W ($P = 0.0054$ and $P = 0.0002$, respectively); SNAP at 0.125, 0.25 and 0.5% W/W ($P = 0.0117$, $P = 0.0123$ and $P = 0.0009$, respectively); pentoxifylline at 5% W/W ($P = 0.0003$); and lisofylline at 0.09, 0.125 and 0.25% W/W ($P = 0.0128$, $P = 0.0001$ and $P = 0.0016$, respectively). Application of ointment base alone (vehicle) was without effect on ipsilateral PWTs for every agent tested (data not shown).

Combination of α_{2A} receptor agonists or NO donors with either PDE or PA inhibitors dramatically reduced the doses required to relieve allodynia in CPIP rats. Thus, the combination of a sub-active dose of clonidine (0.0075% W/W) with pentoxifylline increased PWTs at 0.6 and 1.2% W/W of pentoxifylline ($P = 0.0001$ and $P = 0.0009$, respectively; Fig. 2A), and the pentoxifylline log dose-response x-intercept shifted from 1.572 ± 1.114 mg to 0.2919 ± 0.178 mg ($P = 0.0418$; Fig. 2B). Combining a sub-active dose of linsidomine (0.4% W/W) with lisofylline increased PWTs over pre-drug values at 0.0625 and 0.0932% W/W of lisofylline ($P = 0.0227$ and $P = 0.0315$, respectively; Fig. 2C), and shifted the x-intercept value of the log dose-response curve for lisofylline from a dose of 0.093 ± 0.011 mg to 0.059 ± 0.010 mg ($P = 0.0406$; Fig. 2D). When administered with a sub-active dose of SNAP (0.0625% W/W), lisofylline was anti-allodynic at 0.063% W/W ($P = 0.0096$; Fig. 2E), and the value of the x-intercept of the log dose-response curve for lisofylline shifted from 0.077 ± 0.013 mg to 0.012 ± 0.004 mg ($P = 0.0010$; Fig. 2F). Note that sub-active doses of the α_{2A} receptor agonists or NO donors were selected from the results of single agents presented in Fig. 1.

Application of the most effective drug combinations to the contralateral paw was without effect on the PWTs measured from the injured paw, when compared to pre-drug values. PWTs were thus significantly lower after contralateral paw treatment than after ipsilateral ointment application to the injured paw following treatment with clonidine (0.0075% W/W) + pentoxifylline (0.6% W/W) ($P < 0.0001$; Fig. 3A), linsidomine (0.4% W/W) + lisofylline (0.09% W/W) ($P = 0.0029$; Fig. 3B) or SNAP (0.0625% W/W) + lisofylline (0.0625% W/W) ($P = 0.0001$; Fig. 3C). In addition, for all combinations tested, application of vehicle

(ointment base) to the ipsilateral paw was without effect on the PWTs measured from the injured paw, when compared to pre-drug values.

Effects of topical treatments on mechanical allodynia in rats with CCI, PDN or CIPN

CCI rats developed unilateral allodynia in the hind paw of the injured hind limb. Low concentration combinations of four of the formulations reduced allodynia in CCI rats. These effects were due to a local effect, as the effects were produced by ipsilateral, but not contralateral treatment. Thus, application of clonidine (0.0075% W/W) + pentoxifylline (0.6% W/W) (Fig. 4A), apraclonidine (0.005% W/W) + lisofylline (0.03125% W/W) (Fig. 4B), linsidomine (0.4% W/W) + pentoxifylline (0.3% W/W) (Fig. 4C) or SNAP (0.0625% W/W) + lisofylline (0.0625% W/W) (Fig. 4D) to the injured paw increased PWTs above pre-drug values ($P = 0.0024$, $P = 0.0008$, $P = 0.0111$, and $P = 0.0009$, respectively), and increased PWTs above values measured after ipsilateral vehicle application ($P = 0.0024$, $P = 0.0001$, $P = 0.0018$, and $P = 0.0005$, respectively). Application of the same combinations to the contralateral hind paw were without effect when compared to pre-drug values, and PWTs obtained after ipsilateral application were higher than those measured after contralateral application for clonidine (0.0075% W/W) + pentoxifylline (0.6% W/W) ($P = 0.0013$), linsidomine (0.4% W/W) + pentoxifylline (0.3% W/W) ($P = 0.0018$) and SNAP (0.0625% W/W) + lisofylline (0.0625% W/W) ($P = 0.0004$) (Fig. 4A–D).

PDN rats developed symmetrical bilateral allodynia in both hind paws. Three low dose combinations reduced mechanical allodynia in the treated (ipsilateral), but not untreated (contralateral), hind paw of PDN rats, without altering paw-withdrawal thresholds of age-matched control rats. Combination of apraclonidine (0.0075% W/W) and pentoxifylline (0.6% W/W), increased diabetic PWTs above pre-drug values when measured at 20, 50 and 90 min following drug application ($P = 0.0184$, $P < 0.0001$ and $P = 0.0027$, respectively). PWTs following ointment application were higher than PWTs obtained from the untreated hind paw of diabetic animals at 20, 50 and 90 min ($P = 0.0057$, $P = 0.0012$ and $P = 0.0063$, respectively) (Fig. 5A, B). A combination of apraclonidine (0.005% W/W) + lisofylline (0.0315% W/W) increased PWTs above pre-drug values when measured at 20, 50 and 90 min following drug application ($P = 0.0167$, $P < 0.0001$ and $P = 0.0023$, respectively, Fig. 5C, D), also producing higher PWTs compared to untreated diabetic PWT values at 20, 50 and 90 min post application ($P = 0.0076$, $P < 0.0001$ and $P < 0.0001$, respectively). Comparable results were obtained with linsidomine (0.4% W/W) + lisofylline (0.075% W/W) which similarly increased diabetic PWTs above pre-drug values when measured at 20, 50 and 90 min following drug application ($P = 0.0011$, $P < 0.0001$ and $P < 0.0001$, respectively), and above untreated diabetic PWT values at 20, 50 and 90 min post application ($P = 0.0015$, $P < 0.0001$ and $P < 0.0001$, respectively) (Fig. 5E, F).

CIPN rats developed symmetrical bilateral allodynia in both hind paws. Two low dose combinations acutely reduced mechanical allodynia in the treated (ipsilateral), while vehicle treatment had no effect in the (contralateral), hind paw of CIPN rats. Thus, on the first day of treatment, apraclonidine (0.005% W/W) + lisofylline (0.03% W/W) increased PWTs above vehicle values when measured at 1, 3 and 6 h following drug application ($P < 0.0001$, $P < 0.0001$ and $P = 0.0243$, respectively, Fig. 6A). The treatment also elevated PWTs above

pre-drug baseline PWTs at 1 and 3 h post-application ($P=0.0209$, $P=0.0016$, respectively, Fig. 6A). A combination of linsidomine (0.4% W/W) + pentoxifylline (0.4% W/W) similarly increased PWTs above vehicle values on the first day of treatment when measured at 1, 3 and 6 h following drug application ($P=0.0001$, $P<0.0001$ and $P=0.0006$, respectively, Fig. 6B). The treatment also elevated PWTs above pre-drug baseline PWTs at 3 h post-application ($P=0.0017$, Fig. 6B).

Chronic daily treatment with apraclonidine (0.005% W/W) + lisofylline (0.03% W/W) also significantly elevated PWTs compared to vehicle treatment or to the pre-drug baseline (day 0), with significant differences between drug and vehicle on days 1, 4, 8, 11 and 15 ($P=0.00014$, $P=0.0015$, $P=0.00008$, $P=0.00014$, $P=0.00008$, respectively), and between drug and baseline on days 1, 8, 11 and 15 ($P=0.0399$, $P=0.0212$, $P=0.0190$, $P=0.0384$, respectively, Fig. 6C). Chronic daily treatment with linsidomine (0.4% W/W) + pentoxifylline (0.4% W/W) also significantly elevated PWTs compared to vehicle treatment or to the pre-drug baseline (day 0), with significant differences between drug and vehicle on days 1, 4, 8, 11 and 15 ($P=0.0002$, $P<0.0001$, $P<0.0001$, $P<0.0001$, $P=0.0001$, respectively) and between drug and baseline on days 1, 4, 8, 11 and 15 ($P=0.0138$, $P=0.0170$, $P=0.0003$, $P=0.0186$, $P=0.0075$, respectively, Fig. 6D).

Discussion

Most pharmacological therapies for CRPS-I and neuropathic pain include oral systemic treatments (NSAIDs, opioids, NSAIDs, pregabalin, etc.) that cause significant side-effects, which can include nausea, confusion, dizziness, stomach bleeding, constipation and dependence^{25,65,71}. These side-effects hinder the ability to use therapeutically effective dose levels and reduce patient compliance⁵⁵. Use of topical agents in low doses should avoid these side-effects. With topical treatment, drug concentrations are higher at local target sites, but plasma concentrations are typically less than 10% of the same dose given orally³⁶. Furthermore, topical treatment avoids gastrointestinal (GI) tract and hepatic first pass metabolism, and allows more drug to be active locally with less potential liver or GI toxicity⁹.

In CIPR rats, the topical combinations we have tested produce similar effects as 5 to 55 times higher systemic doses of the individual agents alone, and have efficacy greater than systemic acetaminophen, ibuprofen, dexamethasone or amitriptyline⁶⁷. They also produce maximal effects in both animal models of CRPS and neuropathic pain that are equivalent to those produced by systemic doses of morphine and pregabalin^{54,67}.

We have previously shown that CIPR rats exhibit both enhanced vasoconstriction or vasospasm in response to close intra-arterial NE administration, as well as having evidence of slow flow/no-reflow, indicated by capillary endothelial cell swelling, poor microvascular perfusion and reduced oxygenation in hind paw muscle^{56,93}. Furthermore, hind paw injection of various vasoconstrictive agents including NE, endothelin-1/2 and vasopressin, as well as the endothelial nitric oxide inhibitor, N5-(1-iminoethyl)-L-ornithine dihydrochloride, produce enhanced painful responses in CIPR rats, likely because these vasoconstrictors exacerbated the poor microvascular perfusion in CIPR hind paws^{67,93}. Indeed, we have

shown that the painful responses to hind paw NE injection in CPIP rats are dose-dependently and significantly reduced by systemic administration of either an α_{2A} receptor antagonist or an NO donor⁹³, both of which would increase peripheral vasodilation. We have also recently shown that systemic administration of the PDE4 inhibitor pentoxifylline reduces both allodynia and microvascular (endothelial cell) dysfunction, as measured by post-occlusion reactive hyperemia⁷⁵. Importantly, post-occlusive reactive hyperemia (dependent on nitric oxide release from microvascular endothelial cells) is significantly reduced in CPIP rats, and this endothelial dysfunction is alleviated following systemic pentoxifylline administration⁷⁵. These various signs of microvascular dysfunction and vasospasm and poor perfusion-related pain responses suggest that pain in CPIP rats, and possibly also CRPS, would benefit from topical treatments aimed at increasing local blood flow and reducing microvascular dysfunction.

It has previously been shown that CCI rats, and rats with sciatic nerve lesions, exhibit enhanced xanthine oxidase in sciatic nerve⁴⁷ and reduced vasodilatory responses to either the neuropeptides substance P and CGRP or the nitric oxide donor sodium nitroprusside applied at the base of a skin blister on the foot pad^{2,3,47}. These findings indicate that nerve injury can induce endoneurial oxidative stress and diminished vascular reactivity in hind paw skin. These authors also showed that systemic treatment with antioxidants improved the skin vascular reactivity in CCI rats, as well as reducing concomitant thermal hyperalgesia⁴⁷. Sciatic nerve lesion has also been shown to produce microvascular disturbances in rats, including capillary stasis (decreased blood flow) and increased venular permeability³¹. Importantly, both the microvascular disturbances and signs of deafferentation pain in these rats were reduced by prophylactic systemic administration of clonidine⁵³ or pentoxifylline³². Once again, the reduced blood flow and microvascular dysfunction evidenced in rats with nerve lesions suggests that our topical treatments may be useful for neuropathic pain.

It has been well established that STZ diabetic rats have evidence of microvascular dysfunction, including reduced blood flow in nerve^{42,48} and skin⁴⁵. Importantly, the reduced blood flow^{42,45,48}, and associated tactile allodynia and thermal hyperalgesia⁴², are alleviated by systemic treatment with antioxidants, PDE inhibitors or rheologic agents that reduce blood clotting. STZ rats also exhibit increased platelet aggregation²⁴, and thickening of capillary walls and basement membrane of endoneurial blood vessels⁸⁴. Importantly, both platelet aggregation²⁴ and associated mechanical hyperalgesia¹⁶, are alleviated by the α_{2A} receptor agonist clonidine, while endoneurial capillary abnormalities are reduced following systemic administration of the PDE inhibitor cilostazol⁸⁴. These findings again suggest that topical treatments that increase blood flow and reduce microvascular dysfunction would be useful for treating PDN.

Growing evidence also suggests that there is microvascular dysfunction in rats with CIPN. Endoneurial blood flow is markedly reduced in association with nerve degeneration in rats treated with the chemotherapeutic agents cisplatin⁴⁹ or taxol⁵⁰. Importantly, endoneurial blood flow is increased and nerve degeneration is reduced in CIPN rats by increasing the level of vascular endothelial growth factor, which promotes angiogenesis^{49,50}. Recent evidence also shows that mechanical allodynia and thermal hyperalgesia in CIPN rats can be alleviated by systemic treatment with agents that reduce reactive oxygen²⁰ and nitrogen²².

species, both of which are elevated in association with microvascular dysfunction. Clinically, in addition to CIPN it has often been reported that chemotherapy induces Raynaud's phenomenon^{4,5,86}, a condition where patients develop poor capillary blood flow in the fingers and toes. Evidence indicates that this complication of chemotherapy can be treated with spinal cord stimulation⁸⁶ (which increases peripheral blood flow) or with PDE5 inhibitors, which were shown to increase blood flow⁴. The findings of microvascular abnormalities associated with CIPN again suggest that topical treatments that increase blood flow and reduce microvascular dysfunction would be therapeutically useful for CIPN.

The concentrations of topical agents used in our combinations are much lower than the recommended concentrations used for neuropathic/ischemic pain or other clinical uses, typically clonidine (0.1–0.3%), apraclonidine (0.5–1.0%), linsidomine (2%), pentoxifylline (5–15%), and lisofylline (0.5–5%). Thus, the combinations of these agents used resulted in significant anti-allodynic effects at topical concentrations that are 5 to 640 times lower than those used typically for the single agents. The finding that these combinations produced effects when given to the hind paw ipsilateral, but not contralateral, to injury in both CPIP and CCI rats, and had no effects on allodynia in the untreated hind paw of PDN and CIPN rats, suggests that the agents produce their anti-allodynic effects locally. Thus, by using combinations that greatly potentiate anti-allodynic effects at a local site, it is possible to use even lower topical drug concentrations that are less likely to produce adverse side-effects. Also, combinations that employ apraclonidine, which does not cross the blood brain barrier⁸³, would further reduce the potential systemic side-effects of α_{2A} receptor agonists, which lower blood pressure largely by actions at α_{2A} and/or imidazoline receptors in the brain stem⁸⁸. Furthermore, although the half-life of some of the agents when given systemically is fairly short (e.g., pentoxifylline: 0.84 h; linsidomine: 1.0 h)^{80,81}, slow skin penetration after topical administration is known to produce a prolongation of drug half-life by 5–10 fold²⁶. This likely explains our finding that topical treatment with combinations including these agents produced anti-allodynic effects lasting up to 6 hours in CIPN rats, and why the combinations were effective when tested 3 hours post-application during chronic dosing trials in CIPN rats. Finally, the ability of these combinations to produce equivalent anti-allodynic effects each testing day during chronic dosing, indicates that these agents do not produce tolerance, at least over the 15 days of treatment in CIPN rats.

Our conclusion must be tempered by the known non-specific effects of the agents we used. For example, clonidine acts at imidazoline receptors as well as α_{2A} receptors⁸⁸, and pentoxifylline is a non-specific PDE inhibitor that produces anti-inflammatory effects by interacting with various biological systems. Furthermore, although lisofylline is a phosphatidic acid inhibitor, it is a metabolite of pentoxifylline^{62,76}, and shares many of pentoxifylline's PDE inhibitor activities, including inhibition of various cytokines⁷⁶. However, topical administered clonidine is unlikely to have significant effects at brainstem imidazoline receptors, especially at the low concentrations we have used. Also, clonidine's anti-allodynic effects were mimicked by apraclonidine, which does not cross the blood brain barrier⁸³. In addition, many of the various anti-inflammatory activities of pentoxifylline and lisofylline are linked with their vascular effects^{7,62,76,91}, as microvascular function is key to various processes involved in the inflammatory response, including plasma extravasation, platelet aggregation, leukocyte migration, cytokine activation, among others.

In conclusion, combining topical agents designed to enhance local blood flow and reduce microvascular dysfunction, produce significant anti-allodynic effects in animal models of CRPS-I, neuropathic pain due to nerve constriction, PDN and CIPN. Furthermore, the dramatic potentiation of anti-allodynic effects of PDE and PA inhibitors when combined with α_{2A} receptor agonists or NO donors, should reduce the potential systemic side-effects of these low concentration topical treatments. Acute time course and chronic daily dosing trial in CIPN rats also indicate that these combinations produce long-lasting effects without exhibiting drug tolerance.

Acknowledgments

This research was supported by grants from the Louise and Alan Edwards Foundation, Canadian Institutes of Health Research (MOP-143406), and Natural Sciences and Engineering Research Council of Canada (194525-09) to TJC.

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Fig. 1.

Assessment of the effects of single topical agents clonidine, apraclonidine, linsidomine, SNAP, pentoxifylline and lisofylline (A–F) on paw-withdrawal thresholds (PWTs) to von Frey stimulation of the ipsilateral (injured) hind paw in day 2–14 CPIP rats. Singly, each agent produces dose-related anti-allodynic effects, with higher concentrations producing significant elevations of PWTs and the lowest concentrations failing to produce significant anti-allodynic effects. * $P < 0.05$ between pre- and post-drug mean PWTs.

Fig 2.

Assessment of the effects of topical combinations of pentoxifylline or lisofylline given with either vehicle or ineffective concentrations of clonidine (A,B), linsidomine (C,D) and SNAP (E,F) on paw-withdrawal thresholds (PWTs; A,C,E) and anti-allodynic (PWT) pentoxifylline or lisofylline dose-response curves alone or in combination with clonidine, linsidomine or SNAP (B,D,F) in the ipsilateral (injured) hind paw of day 2–14 CPIP rats. The combinations significantly increased PWTs at concentrations much lower than in Fig. 1, and shifted the anti-allodynic dose-response curve for lisofylline between 2 and 10 fold to the left. * $P < 0.05$ between pre- and post-drug mean PWTs.

Fig. 3.

Comparison of the effects of topical vehicle application, or ipsilateral versus contralateral application of the most effective concentrations of the topical combinations used in Figs. 2 (clonidine + pentoxifylline (A), linsidomine + lisofylline (B) and SNAP + lisofylline (C)) on paw-withdrawal thresholds (PWTs) in the ipsilateral (injured) hind paw of day 2–14 CIPIP rats. In all trials, topical application of vehicle failed to significantly alter PWTs.

Furthermore, although each combination significantly increased PWTs when applied to ipsilateral hind paw, they were all ineffective when applied to the contralateral hind paw. * $P < 0.05$ between pre- and post-drug mean PWTs; † $P < 0.05$ from post-drug vehicle; § $P < 0.05$ from post-drug contralateral PWT.

Fig. 4.

Comparison of the effects of topical vehicle administration, or ipsilateral versus contralateral administration of the effective concentrations of four topical combinations (clonidine + pentoxifylline (A), apraclonidine + lisofylline (B) linsidomine + pentoxifylline (C) and SNAP + lisofylline (C)), on paw-withdrawal thresholds (PWTs) in the ipsilateral (injured) hind paw of day 7–14 CCI rats. In all trials, topical application of vehicle failed to significantly alter PWTs. Furthermore, although each combination significantly increased PWTs when applied to ipsilateral hind paw, they were all ineffective when applied to the contralateral hind paw. $*P < 0.05$ between pre- and post-drug mean PWTs. $^{\dagger}P < 0.05$ from post-drug vehicle. $^{\ddagger}P < 0.05$ from post-drug contralateral PWT.

Fig. 5.

Time course of the anti-allodynic effects of low concentration topical combinations of apraclonidine + pentoxifylline (A,B), apraclonidine + lisofylline (C,D) and linsidomine + lisofylline (E,F) on paw-withdrawal thresholds (PWTs) both ipsilateral and contralateral to the topical treatments in rats with streptozotocin (STZ)-induced diabetic neuropathy (A,C,E) or in age-matched vehicle-injected controls (B,D,F). Each topical combination significantly increased PWTs of the ipsilateral (treated), but not the contralateral (untreated) hind paws of rats with diabetic neuropathy. Conversely, the topical combinations had no effects on PWTs in the ipsilateral or contralateral hind paws in age-matched (STZ vehicle-injected) control rats. * $P < 0.05$ between pre- and post-drug mean PWTs within a group. † $P < 0.05$ between

ipsi- and contralateral PWTs, and $P < 0.05$ between diabetic and control PWTs at each time point.

Fig. 6.

Anti-allodynic effects over an acute time course (A,B) and chronic daily dosing (C,D) of topical combinations of apraclonidine + lisofylline (A,C) or linsidomine + pentoxifylline (B,D) in rats with CIPN. Each combination produced acute anti-allodynic effects lasting 6 h after the first topical application (* $P < 0.05$ compared to vehicle, † $P < 0.05$ compared to pre-drug baseline (0)), and produced significant anti-allodynia on each test day during chronic daily dosing for 15 days (* $P < 0.05$ compared to vehicle, † $P < 0.05$ compared to pre-drug baseline (0)).