Topical Combinations to Treat Microvascular Dysfunction of Chronic Postischemia Pain

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Abstract

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Attestation: AL attests to the integrity of the original data and the analysis reported in this manuscript and is the archival author.

Conflicts of Interest: Patent, potential commercialization.

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Contribution: Animal testing.

Conflicts of Interest: The author has no conflicts of interest to declare.

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Contribution: Animal testing.

Conflicts of Interest: The author has no conflicts of interest to declare.

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Conflicts of Interest: Patent, potential commercialization.

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Contribution: Study design, figure & manuscript preparation.

Attestation: TJC attests to the integrity of the original data and the analysis reported in this manuscript.

Conflicts of Interest: Patent, potential commercialization.

This manuscript was handled by: Martin S. Angst, MD

Background—Growing evidence indicates that patients with complex regional pain syndrome (CRPS) exhibit tissue abnormalities caused by microvascular dysfunction in the blood vessels of skin, muscle and nerve. We tested whether topical combinations aimed at improving microvascular function would relieve allodynia in an animal model of CRPS. We hypothesized that topical administration of either α_2 -adrenergic (α_2 A) receptor agonists or nitric oxide (NO) donors given to increase arterial blood flow, combined with either phosphatidic acid (PA) or phosphodiesterase (PDE) inhibitors to increase capillary blood flow, would effectively reduce allodynia and signs of microvascular dysfunction in the animal model of chronic pain.

Methods—Mechanical allodynia was induced in the hind paws of rats with chronic postischemia pain (CPIP). Allodynia was assessed before and after topical application of vehicle, single drugs or combinations of an α_2 A receptor agonist (apraclonidine) or an NO donor (linsidomine), with PA or PDE inhibitors (lisofylline, pentoxifylline). A topical combination of apraclonidine + lisofylline was also evaluated for its effects on a measure of microvascular function (post-occlusive reactive hyperemia) and tissue oxidative capacity (formazan production by tetrazolium reduction) in CPIP rats.

Results—Each of the single topical drugs produced significant dose-dependent antiallodynic effects compared to vehicle in CPIP rats ($n = 30$), and the antiallodynic dose-response curves of either PA or PDE inhibitors were shifted 5 to 10 fold to the left when combined with nonanalgesic doses of α_2 A receptor agonists or NO donors ($n = 28$). The potent antiallodynic effects of ipsilateral treatment with combinations of α_2 A receptor agonists or NO donors with PA or PDE inhibitors, were not reproduced by the same treatment of the contralateral hindpaw ($n = 28$). Topical combinations produced antiallodynic effects lasting up to 6 h ($n = 15$), and were significantly enhanced by low dose systemic pregabalin in early, but not late, CPIP rats ($n = 18$). An antiallodynic topical combination of apraclonidine + lisofylline was also found to effectively relieve depressed post-occlusive reactive hyperemia in CPIP rats ($n = 61$), and to increase formazan production in postischemic tissues (skin and muscle) ($n = 56$).

Conclusions—The present results support the hypothesis that allodynia in an animal model of CRPS is effectively relieved by topical combinations of α_2 A receptor agonists or NO donors with PA or PDE inhibitors. This suggests that topical treatments aimed at improving microvascular function by increasing both arterial and capillary blood flow produce effective analgesia for CRPS.

Introduction

Complex regional pain syndrome (CRPS) typically follows fracture, crush or soft tissue injury, with (type II) or without (type I) accompanying injury of a major nerve. CRPS-I (formerly known as reflex sympathetic dystrophy) and CRPS-II (formerly known as causalgia) have historically been viewed to depend on pathology in the sympathetic nervous system. However, there is evidence to suggest that a key mechanism underlying the pathology of both CRPS-I and CRPS-II is persistent deep tissue ischemia associated with microvascular dysfunction.^{1–5} We have generated evidence that pain in an animal model of CRPS also depends on microvascular dysfunction.^{6–9} In rats with chronic postischemia pain (CPIP), whose symptoms parallel those of patients with CRPS, ischemic tissue injury leads to the generation of oxygen free radicals and proinflammatory cytokines, which causes arterial vasospasm and capillary slow flow/no-reflow (signs of microvascular dysfunction) in

hindpaw blood vessels.^{6–9} Vasospasm and capillary slow flow/no-reflow lead to reduced nutritive blood flow, poor muscle oxygenation and the build-up of muscle lactate, all of which contribute to the pain.^{7,8} Thus, CIP animals have pain, allodynia, vasospasm, poor tissue perfusion, and oxidative stress in their affected hindpaw.⁸ Importantly, we showed that pain/allodynia and microvascular dysfunction in CIP rats are attenuated by systemic treatments with an α_2 A receptor agonist and nitric oxide (NO) donors, which improve arterial blood flow (relieving vasospasm), and by a phosphodiesterase (PDE)4 inhibitor (pentoxifylline), which improves capillary blood flow (relieving capillary slow flow/no-reflow).^{7–9} Therefore, we hypothesized that treatments aimed at enhancing tissue oxygenation by reducing arterial vasospasm and capillary slow flow/no-reflow will effectively relieve pain in this animal model of CRPS, and potentially in CRPS patients.

Regional blood flow is regulated by the vasoconstrictive transmitter norepinephrine (NE) released from sympathetic postganglionic neurons, which contracts vascular smooth muscles.^{10,11} Blood flow is also regulated by the vasodilatory substance NO that is released from vascular endothelial cells and relaxes vascular smooth muscles.^{12,13} Thus, drugs that reduce NE release or binding, or drugs that increase NO, can be used to alleviate conditions with poor blood flow,^{14,15} including microvascular dysfunction. Various isoforms of PDE enzymes (types 1–11) also regulate blood flow, and affect various rheological properties, by influencing the intracellular messengers cAMP and cGMP.¹⁶ Thus, PDE4,7 and 8 hydrolyse cAMP, PDE5,6 and 9 hydrolyse cGMP, and PDE1,2,3,10 and 11 hydrolyse both cAMP and cGMP.¹⁷ While PDE inhibitors that reduce cGMP hydrolysis enhance blood flow,¹⁸ PDE inhibitors that reduce cAMP hydrolysis also reduce platelet aggregation,¹⁹ decrease blood viscosity,²⁰ and increase the flexibility of red blood cells,²⁰ all of which relieve microvascular dysfunction. Thus, PDE inhibitors have been developed for conditions such as peripheral arterial disease²¹ and intermittent claudication,²⁰ where microvascular injury leads to capillary low flow/no reflow and reduced tissue oxygenation. Pentoxifylline, a PDE4 inhibitor, has been specifically developed to treat these ischemic disorders.^{20,21} Lisofylline, a major metabolite of pentoxifylline, shares many of the actions of its prodrug,²² but also inhibits the conversion of lysophosphatidic acid to phosphatidic acid (PA), a pathway critical to the production of various cytokines, including interleukin 1β and tumor necrosis factor- α .²³

Since systemic use of drugs that regulate blood flow may seriously affect arterial blood pressure and cardiac function, the topical use of these drugs could enhance both their efficacy and safety. Also, since the pain of CRPS may depend not only on reduced arterial blood flow, but also microvascular dysfunction at the capillary level, pain relief may be optimized by the simultaneous use of drugs that increase both arterial and capillary blood flow; that is by combining α_2 A receptor agonists or NO donors with PDE or PA inhibitors. Although α_2 A receptor agonists,^{24,25} and NO donors^{26,27} have been used topically or regionally to alleviate chronic pain, the topical pain-relieving effects of PDE and PA inhibitors have not been previously assessed. Furthermore, these drugs have not been combined systemically, or topically for the treatment of chronic pain. However, as single drugs, α_2 A receptor agonists, NO donors or PDE inhibitors have been used systemically to treat pain associated with angina,^{28–30} peripheral arterial disease^{31,32} and neuropathic pain,^{33–35} indicating their usefulness in these syndromes. PA inhibitors share the vasodilator,³⁶

antiischemic³⁷ and antiplatelet aggregation³⁸ effects of PDE inhibitors, but their added antioxidant,³⁹ anticytokine,⁴⁰ antileukocyte attractant,⁴¹ immunosuppressant,⁴² and mitochondrial protective⁴³ effects suggest they may be more effective at relieving chronic pain.

In the present study, we have tested the hypothesis that combinations of drugs aimed at reducing microvascular dysfunction (including α_2 A receptor agonists or NO donors, combined with PA or PDE inhibitors) can be used topically on the hindpaw to reduce allodynia in an animal model of CRPS. One combination was also tested for its ability to restore compromised post-occlusive reactive hyperemia (PORH) that is observed after ischemia-reperfusion (I/R) injury in CPIP rats.

Methods

Animals

Male Long Evans rats (225–250 g, Charles River, St. Constant, QC) were received at our animal housing facility at least 7 days before the experiments started. Our study 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 for topical administration included linsidomine (Tocris, Ellisville, MO), lisofylline (Cayman Chemical, Ann Arbor, MI), apraclonidine and pentoxifylline (Sigma-Aldrich, St. Louis, MO). Pregabalin, used for systemic treatment, was obtained as a gift from Pfizer corporation.

Analgesic formulations

Ointment-type analgesic formulations containing the above-mentioned drugs were formulated using a composite, water-soluble polyethylene glycol base system using carbowax (PEG 3350) and PEG 400 (both from Sigma-Aldrich, St. Louis, MO) in the ratio of 60:40 respectively. The water-soluble base system was selected due to its non-sticky nature. Briefly, the required amounts of the active ingredients were first weighed 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 $\pm$ 2.7 mg) of the ointment was used for all rat hindpaw applications throughout the experimental procedure.

CPIP

CPIP was generated after prolonged hindpaw ischemia and reperfusion, as previously described by Coderre et al.⁶ Briefly, rats were anesthetized over a 3 h period with a bolus (55 mg/kg, i.p.) followed by 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 hindlimb proximal to the ankle joint. The tight-fitting O-ring produces a complete

blockade of arterial blood flow as measured using laser Doppler flowmetry⁸ (also see Fig. 1). The ring was then left in place for 3 h, and the rats recovered from anesthesia 30–60 min after reperfusion. Sham rats were anesthetized for the same period and had a cut (loose) O-ring placed around their ankles. Rats were tested either between 2 and 14 (early) or 18–26 days (late) post-I/R or sham injury.

Mechanical sensitivity testing

The plantar surface of the ipsilateral hindpaw was tested for mechanical allodynia (paw withdrawal thresholds, PWTs) in CPIP rats. Semmes Weinstein nylon monofilaments 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. Six different filaments were used: the minimum stimulus intensity was 1 g and the maximum was 15 g. Individual threshold determinations could thus require from 2–6 filaments and from 6–9 stimuli, depending on the pattern of responses obtained. Each filament was applied for 10 s or until a flexion reflex occurred. 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 $(10^{[Xf+k\delta]})/10,000$ where Xf= 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.⁴⁴).

Pharmacological treatments

The topical formulations containing an NO donor, α_2 A receptor agonist or a PA/PDE inhibitor were tested for their antiallodynic effects, either singly, or in combination with each other, at concentrations selected from the published literature for use as topical drugs. In the single drug pharmacological trials, apraclonidine was tested 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); lisofylline at 0.063, 0.09, 0.125 and 0.25% W/W (n = 10); and pentoxifylline at 0.6, 1.2, 2.5 and 5% W/W (n = 6). Each drug was tested in a separate group. Doses were tested in ascending fashion with a minimum of 24 h washout period between each successive test. The absence of carryover drug effects was confirmed by comparing pre-drug measurements for each group to those of the preceding trial.

In separate groups of rats, formulations containing lisofylline or pentoxifylline were tested in drug combinations with noneffective concentrations of apraclonidine or linsidomine. Accordingly, the formulations tested in the combination trials included apraclonidine (0.005% W/W) + lisofylline (0.0078, 0.0156 and 0.0313% W/W) (n = 6) and linsidomine (0.4% W/W) + pentoxifylline (0.075, 0.15 and 0.3% W/W) (n = 8).

A third cohort of rats was used to confirm the local site of action of the topical treatments. For this study, the formulations were applied to the contralateral paw (noninjured) and the ipsilateral paw (injured) was tested for antiallodynic effects. The most effective drug combinations were tested in this manner. Accordingly, the combinations included apraclonidine (0.005% W/W) + lisofylline (0.03125% W/W) (n = 6), and linsidomine (0.4% W/W) + pentoxifylline (0.3% W/W) (n = 8). 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 after application. CPIP rats treated with apraclonidine (0.005% W/W) + lisofylline (0.0156% W/W) or linsidomine (0.4% W/W) + lisofylline (0.0318% W/W) were also assessed to compare pre-drug PWTs and the time course of drug effect on PWTs between 20 min to 24 h after treatment. Also, pre- and post-drug PWTs were assessed in either early or late CPIP rats that were treated with systemic pregabalin (10 mg/kg in sterile saline, i.p.), topical apraclonidine (0.005% W/W) + lisofylline (0.03% W/W), or both systemic pregabalin and the topical combination at the same doses at 120 min post-drug for all treatments.

Laser Doppler flowmetry

Plantar blood flow (flux) was assessed using a laser Doppler perfusion and temperature monitor (DRT4, Moor Instruments, Wilmington, DE) with the laser-emitting probe (7.0 mm in diameter) placed between the tori pads along the midline of the plantar hindpaw. Briefly, rats were anesthetized by i.p. administration of a bolus of urethane (30% w/v in saline at a dose of 0.3 mL/100 g body weight) and α -chloralose (1% w/v in water at a dose of 0.125 mL/100 g body weight). α -chloralose was initially dissolved at 55°C. Body temperature was monitored throughout the experiment by a rectal thermometer coupled to a heating pad (FHC, Bowdoinham, ME), and was maintained between 36.0°C and 39.0°C. Deep anesthesia, defined by the absence of spontaneous vibrissae movement, pedal reflexes and blink responses was achieved at normal heart rate (monitored by the flux probe) and normal respiratory rate (range: 240–310 bpm and 80–110 breaths per min, respectively). Anesthesia level was maintained throughout the experiment by the i.p. administration of additional anesthetic mixture at half the original dose, as required.

PORH

Plantar blood flow of the hindpaw was monitored in the left hindpaw of rats previously subjected to the I/R injury (CPIP), or of the left paw of sham-injured control animals (Sham CPIP). All CPIP animals were tested between 5 and 10 days after the initial I/R or sham injury. Before recording plantar blood flow, each rat underwent a period of stabilization lasting 25 to 45 minutes. An initial 2-minute period of baseline blood flux was recorded, followed immediately by a 2-minute occlusion induced by pressurizing and inflating a tight-fitting loop of Tygon® R 3603 tubing (outside diameter = 2.38 mm, inside diameter = 0.79 mm, wall thickness = 0.79 mm) connected to an air-filled pump-driven 60 mL syringe (Model 11, Harvard Apparatus, Montreal, QC), creating a tourniquet around the ankle joint. Ischemia was confirmed by observing a flux reduction more than 95% of preocclusion value. Two minutes later, the pressure was released quickly and the tourniquet loosened to allow reperfusion to occur. Pre-drug PORH was monitored by sampling flux at the rate of 1/sec for 2 min after the onset of reperfusion (Fig. 1 for a sample trace). The laser Doppler probe was then removed and a topical combination of 0.01% apraclonidine and 0.06% lisofylline was applied to the hindpaw, as above. The plantar probe was then replaced, and recording resumed. Beginning 45 minutes after ointment application, a second series of baseline recording, 2 min occlusion, reperfusion, and 2 min recording of the post-drug PORH was performed. Rats were euthanized immediately after the second PORH trial.

Analysis of oxidative capacity in muscle and skin by spectrophotometric measurement of tetrazolium reduction

After pentobarbital overdose, muscle samples of the superficial plantar layer (one each from the Flexor Hallucis Brevis, Flexor Digiti Minimi Brevis and Flexor Digitorum Brevis) weighing between 44 and 70 mg (mean 55.8 ± 2.2 mg), and approximately 0.7 X 0.8 cm samples of the glabrous plantar skin weighing between 39 and 47 mg (mean 44.8 ± 4.7 mg) were collected from the postischemic and contralateral hindpaws of treated and from the left and right hindpaws of sham-injured rats 60 min after the application of either vehicle (ointment base) or a topical combination of 0.01% apraclonidine and 0.06% lisofylline to the postischemic hindpaw of treated animals or the left hindpaw of sham-injured rats. Samples were collected on ice, sliced longitudinally with a scalpel blade and immediately incubated in a solution of 10% 2,3,5-triphenyltetrazolium chloride in phosphate buffered saline pH 7.4 for 60 min at 37°C. The samples were then mechanically homogenized in 0.25 M sucrose in phosphate buffered saline (100 µl/mg wet weight) and centrifuged at 1000 g for 5 min. The supernatant was collected, its protein content was assayed by the Bradford method and was then incubated in acetone (2:1 V/V) at 37°C for 40 min with occasional agitation to extract the formazan produced by reduction of the tetrazolium salt. After a final centrifugation at 1000 g for 10 min, 200 µl of supernatant was collected from each processed sample and its absorbance at 485 nm was measured in a spectrophotometer. Background was measured from samples processed without tetrazolium. Net absorbance was calculated by background subtraction and divided by total sample protein.

Data analysis

Calculated (von Frey) PWTs were averaged by group, treatment time and dose. The data were first assessed for normality (Shapiro-Wilk test) and variance homogeneity (Levene test) and subjected to analysis of variance (ANOVA) using repeated measures (pre- vs. post-drug being the first repeated factor and drug dose being the second repeated factor). For single drug trials, drug effects were assessed by pairwise comparisons of group means using Bonferroni tests after the observation of significant interaction effects of drug by dose in the repeated measures ANOVA (pre- vs. post-drug by dose). For control trials testing the effects of applying effective topical combinations to the untreated, contralateral paw, pairwise comparisons of mean PWTs were made using Bonferroni tests after the observation of significant drug by dose interaction effects.

Shifts in drug antiallodynic potency obtained by the use of combination treatments were illustrated by first calculating the difference between pre- and post-drug measures for each rat (PWT), 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.

The flux measurements recorded from each animal during the PORH period were first normalized to baseline (expressed as a % change from the mean value of the baseline period

preceding each occlusion) and then averaged into successive 10 sec periods spanning the 120 seconds of reperfusion. These binned data were then grouped by injury and topical drug treatment (sham-vehicle; n=8, sham-drug; n=7, CPIP-vehicle; n=9 and CPIP-drug; n=10). In order to compare the magnitude of drug effects among the treatment groups, the total area under the normalized hyperemic response curve for each rat was calculated for each reperfusion period using the trapezoidal rule. The area under the curve (AUC) measurements were then averaged by treatment group and drug and subjected to a two-way ANOVA with one repeated measure (treatment and pre- vs. post-drug as the second, repeated factor), followed by pairwise orthogonal contrasts. The effect of drug or vehicle application within each treatment group over the reperfusion time was assessed by subjecting the binned flow data from each group to a two-way ANOVA with one repeated measure, followed by *post hoc* pairwise comparisons using the Bonferroni test, when appropriate.

Absorbance measurements were likewise grouped by injury and topical drug treatment (sham-vehicle; n = 8, sham-drug; n = 6, CPIP-vehicle; n = 9 and CPIP-drug; n = 9). The grouped data were first assessed for normality (Shapiro-Wilk test) and variance homogeneity (Levene test) and analysed by three-way ANOVA with one repeated factor: injury (I/R vs sham), drug treatment (drug vs vehicle) and ipsilateral versus contralateral paw (the repeated factor). The observation of a significant interaction effect was followed by *post hoc* pairwise comparisons using a Bonferroni test, when appropriate.

Statistical computations were performed with Statistica, version 6 (Statsoft, Tulsa, OK) or Prism 5 (GraphPad Software, La Jolla, CA). Significance was defined as $P < 0.05$.

Results

Effects of topical treatments on mechanical allodynia in CPIP rats

In the single drug pharmacological trials, topical hindpaw application produced significant, dose-related antiallodynic effects in CPIP rats for each of the four drugs tested (Fig. 2A–F). Apraclonidine significantly elevated PWTs above pre-drug levels at 0.02 and 0.04% W/W ($P = 0.023$ and $P = 0.009$, respectively; Drug by Dose interaction $P = 0.019$); linsidomine at 1.6% W/W ($P = 0.005$; Drug by Dose interaction $P = 0.025$); lisofylline at 0.125 and 0.25% W/W ($P = 0.0008$ and $P = 0.044$, respectively; Drug by Dose interaction $P = 0.038$); and pentoxifylline at 5% W/W ($P = 0.0003$; Drug by Dose interaction $P = 0.002$). Application of ointment base alone (vehicle) was without effect on ipsilateral PWTs for every drug tested (data not shown).

The combination of apraclonidine 0.005% W/W with lisofylline significantly elevated PWTs above pre-drug values at 0.0313% W/W ($P = 0.037$; Pre-drug vs post-drug by Dose interaction $P = 0.012$; Fig. 3A), and shifted the x-intercept value of the log dose-response curve for lisofylline from (mean $\pm$ SEM) 0.154 ± 0.046 mg to 0.012 ± 0.003 mg ($P = 0.0018$; Fig. 3B). When combined with 0.4% W/W linsidomine, pentoxifylline significantly increased PWTs at 0.30 W/W ($P = 0.0232$; pre-drug vs. post-drug by Dose interaction $P = 0.0046$ Fig. 3C). The pentoxifylline log dose-response curve was shifted to the left and the x-intercept dose decreased from 1.318 ± 0.072 mg to 0.542 ± 0.127 mg ($P = 0.001$; Fig. 3D).

Application of 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 ointment application to the injured paw following treatment with apraclonidine 0.005% W/W + lisofylline 0.03125% W/W ($P = 0.014$; Treatment by Drug (pre- vs. post-drug) interaction $P = 0.117$, Fig. 4A) or linsidomine 0.4% W/W + pentoxifylline 0.3% W/W ($P = 0.047$; Treatment by Drug (pre vs. post-drug) interaction $P = 0.005$; Fig. 4B). In addition, for all combinations tested, application of vehicle to the ipsilateral paw was without effect on the PWTs measured from the injured paw, when compared to pre-drug values (for all treatments, Treatment by Drug (pre vs. post-drug) interaction $P = 0.473$).

In time course trials, two low dose combinations produced prolonged reductions in mechanical allodynia in the treated (ipsilateral) hindpaw of CPIP rats. Thus, apraclonidine (0.005% W/W) + lisofylline (0.0156% W/W) increased PWTs above pre-drug baseline values when measured at 45 min after drug application ($P = 0.002$; Effect of time after drug administration $P = 0.0003$), Fig. 5A). A combination of linsidomine (0.4% W/W) + lisofylline (0.0318% W/W) increased PWTs above pre-drug baseline values when measured at 45 min and 3 h after drug application ($P = 0.002$; $P = 0.021$, respectively; effect of time after drug administration $P = 0.0004$ Fig 5B).

Effects of systemic administration of pregabalin along with topical treatments on mechanical allodynia in early vs. late CPIP rats

The effect of pregabalin in early CPIP rats was found to depend on the administration of topical drugs (drug by group interaction $P = 0.0073$). Thus, systemic administration of 10 mg/kg, i.p. pregabalin did not significantly affect allodynia (Fig. 6), with post-pregabalin PWTs remaining unchanged from pre-drug baseline or post-drug vehicle PWTs ($P = 0.996$ and $P = 0.994$, respectively). A higher dose of pregabalin (30 mg/kg i.p.) was found to be significantly antiallodynic at 1 ($P = 0.0007$) and 3 h ($P = 0.011$) after injection (data not shown). However, the noneffective 10 mg/kg dose of systemic pregabalin significantly enhanced the antiallodynic effect of the topical combination of apraclonidine + lisofylline ($P = 0.046$). In early CPIP animals, topical apraclonidine + lisofylline significantly elevated PWTs compared to pre-drug baseline PWTs ($P = 0.025$), and systemic pregabalin given along with the topical treatment significantly elevated PWTs above both pre-drug baseline PWTs ($P = 0.0007$) and post-drug PWTs of the topical treatment alone ($P = 0.025$). In late CPIP animals, pregabalin and the topical treatment had no comparable interaction ($P = 0.104$). Pregabalin was very effective when used alone at 10 mg/kg ($P = 0.010$), but was no more effective when given in addition to the topical treatment ($P = 0.261$ versus pre-drug baseline).

Effects of topical combination treatment on PORH in CPIP and sham rats

In early CPIP rats, all four treatment groups exhibited PORH at the end of each ischemia period, as indicated by a rapid elevation in flow gradually abating towards preocclusion levels over the course of the measurement period (Fig. 7A–D). However, the magnitude of the flow increase (Fig 7A–D) and the changes in AUC (Fig. 8) varied depending on treatment group and topical drug application, as indicated by a significant interaction

between these two variables ($P = 0.019$). This occurred in part because both CPIP groups displayed significantly weaker hyperemic responses to the pre-drug occlusion compared to their controls (CPIP-drug vs. sham-drug: $P = 0.004$ and CPIP-vehicle vs sham-vehicle: $P = 0.029$) (Fig. 8). In addition, drug-treated CPIP animals, but not vehicle-treated CPIP rats displayed significantly increased hyperemic responses to the second ischemic challenge (pre- vs. post-drug: $P = 0.0001$ vs. $P = 0.675$, respectively) (Fig. 8). The PORH AUC measured from sham-injured animals after drug or vehicle application did not change ($P = 0.621$ and $P = 0.676$, respectively) (Fig. 8). A similar pattern was seen in the time course data: only drug-treated CPIP animals displayed a significant increase in flow after drug application ($P = 0.508$, $P = 0.0003$, $P = 0.064$ and $P = 0.174$ for the CPIP-vehicle (Fig 7A), CPIP-drug (Fig. 7B), sham-drug (Fig. 7C) and sham-vehicle (Fig. 7D) groups, respectively). The increase in flow in CPIP animals given apraclonidine and lisofylline was detected from the onset to 80 sec of the reperfusion period. (Fig. 7B).

In contrast to the decreased PORH in early CPIP rats, flux measures displayed a large PORH in late CPIP rats (effect of time since start of reperfusion $P < 0.00001$, data not shown). Also in late CPIP rats, there were no effects of topical drug application compared to vehicle treatment ($P = 0.721$), no effects of injury (CPIP or sham-injury; $P = 0.127$), and no interactions between drug and injury ($P = 0.509$).

Effects of topical combination treatment on skin and muscle oxidative capacity in CPIP and sham rats

In early CPIP and sham groups, formazan production in skin was affected by the presence of an I/R injury and the topical drug treatment (injury by drug by side interaction $P = 0.0182$). Thus skin from the injured hindpaw of CPIP animals treated with vehicle displayed lower levels of formazan than that of their uninjured contralateral hindpaw ($P = 0.0067$), and that of the vehicle-treated hindpaw of sham-injured animals ($P = 0.0179$). Topical application of apraclonidine and lisofylline to the CPIP hindpaw resulted in higher levels of formazan in the skin compared to that in skin samples from vehicle-treated CPIP rats ($P = 0.0039$). Formazan levels measured after drug combination treatment in CPIP hind paw skin did not differ from those obtained from the uninjured contralateral hindpaw skin or from drug- or vehicle-treated hindpaw skin from sham animals ($P = 0.556$). Drug application to the uninjured hindpaw of CPIP rats or to either hindpaw of sham animals was similarly without effect compared to vehicle treatment ($P = 0.992$). Vehicle application was without effect (compared to the contralateral untreated side).

A similar pattern of effects was noted in muscle tissue (injury X drug X side interaction $P = 0.0042$) (Fig. 9). Formazan levels were lower in muscle samples from the I/R-injured hindpaw of CPIP rats compared to those from the uninjured contralateral hindpaw ($P = 0.001$), or to those obtained from sham-injured animals ($P = 0.0146$ and $P = 0.0263$ for sham-injured and contralateral hindpaw, respectively). Topical application of apraclonidine and lisofylline increased formazan levels in CPIP hindpaw muscle to a level similar, not different, from sham-injured hindpaws receiving the topical combination ($P = 0.1061$) or vehicle ($P = 0.3003$). Topical application of apraclonidine and lisofylline to the contralateral hindpaw was without effect in samples from the allodynic hindpaw ($P = 0.8969$), or in

hindpaw muscle samples obtained from sham-injured rats ($P = 0.4599$). Vehicle application was without effect (compared to the untreated contralateral side, $P = 0.9901$) (Fig. 9).

In late CPIP and sham groups, the effects of I/R injury were markedly different: formazan levels in hindpaw skin and muscle did not differ between vehicle-treated CPIP and contralateral samples, nor between vehicle-treated CPIP and sham samples ($P = 0.368$ for all interactions and main effects). Application of an apraclonidine and lisofylline combination at the same concentrations used in early CPIP rats had no effect on late CPIP, vehicle-treated or contralateral samples (Fig 9).

Discussion

In the present study, we show that topical combinations of drugs aimed at reducing microvascular dysfunction synergistically relieve allodynia in CPIP rats. Thus, a combination of α_2 -adrenergic agonists or NO donors with either PDE or PA inhibitors produce antiallodynic effects that are much more potent than the single drugs alone. In CPIP rats, these topical combinations have an antiallodynic efficacy greater than systemic acetaminophen, ibuprofen, dexamethasone or amitriptyline⁴⁵. The topical combinations also produced maximal effects that are equivalent to those produced by systemic doses of morphine and pregabalin.^{45,46} The concentrations of topical drugs used in the present combinations are much lower than the recommended concentrations used for neuropathic/ischemic pain or other clinical uses, which typically use apraclonidine (0.5–1.0%), lisofylline (0.5–5%), pentoxifylline (5–15%) and linsidomine (2%). Thus, the combinations of these drugs resulted in significant antiallodynic effects at topical concentrations that are 5 to 640 times lower than those used typically for the single drugs. Thus, by using combinations that greatly enhance antiallodynic effects, it is possible to use even lower topical drug concentrations that are less likely to produce adverse side effects. Also, combinations that use apraclonidine, which does not cross the blood-brain decrease arterial blood pressure largely by actions at presynaptic α_2A and/or imidazoline receptors in the brainstem.⁴⁸

We also show that one of these combinations (apraclonidine + lisofylline) produced a significant increase of PORH and improved oxidative capacity in the hindpaw skin and muscle of early CPIP rats, which normally have depressed PORH, as well as exhibit poor microvascular perfusion and reduced oxidative capacity in hindpaw muscle.^{7,8} Although topical treatment with apraclonidine + lisofylline significantly increased PORH in CPIP rats, it had no effects in sham rats, while vehicle treatment had no effect in either group. The current results support our earlier findings that systemic administration of the PDE4 inhibitor pentoxifylline reduces both allodynia and microvascular dysfunction, as evidenced by reduced PORH, in CPIP rats.⁹ PORH has been shown to be a robust measure to examine microvascular function,⁴⁹ and has been shown to be reduced in various conditions in which microvascular dysfunction has been suspected.⁵⁰ PORH has also been used to assess microvascular function in CRPS patients. Koban et al.⁵ reported that PORH was reduced in the affected arms of CRPS patients, compared to the contralateral limb or the limbs of control subjects. These results were confirmed by Dayan et al.⁴ who found reduced PORH

in both affected arms and legs of CRPS patients. Moreover, Kurvers et al.¹ also showed that PORH was reduced in the affected limbs of cold (stage II and III) CRPS patients.

Previously, studies exhibiting analgesic effects of topical/regional clonidine in CRPS patients^{24,25} or in rat models of neuropathic pain⁵¹ did not address the role of blood flow or microvascular dysfunction. However, studies^{27,52,53} which used either a topical NO donor or a systemic PDE inhibitor have compared the analgesic effects of these treatments and their effects on blood flow in CRPS patients. Thus, Groeneweg et al.²⁷ showed that a topical ointment of the NO donor isosorbide dinitrate that caused vasodilation, and a 4–6°C increase in skin temperature, produced analgesia in the majority of cold CRPS patients. However, in a second study⁵² these authors reported that the same NO donor applied topically in Vasoline®, which did not affect vasodilation or skin temperature, was unable to reduce pain scores in CRPS patients, suggesting that the topical formulation was suboptimal. In a separate study, Groeneweg et al.⁵³ reported a small 15% reduction in pain scores with a systemic treatment with the PDE inhibitor tadalafil that did not significantly alter skin temperature, relative to placebo, in CRPS patients. While not particularly impressive, none of these studies used combinations of α_2 A receptor agonists or NO donors with PDE or PA inhibitors as we have here, to target both reduced blood flow and microvascular dysfunction.

In the present study, we also found that 10 mg/kg systemic pregabalin was ineffective at relieving allodynia in early CPIP rats, for which hindpaw PORH and oxidative capacity was suppressed, but systemic pregabalin enhanced the efficacy of low dose apraclonidine + lisofylline in early CPIP rats. In late CPIP rats (i.e. more than 18 days after I/R injury), which show normal hindpaw PORH and oxidative capacity, 10 mg/kg of pregabalin was maximally effective, and its antiallodynic effect could not be further enhanced by the topical combination, which itself lacked efficacy in late CPIP rats. These findings suggest that CPIP rats evolve from a phase in which hindpaw microvascular dysfunction is critical, and our topical combinations are most effective at relieving allodynia, to a phase in which hindpaw microvascular dysfunction has resolved and systemic pregabalin is more effective. We expect that allodynia during this second phase is dependent on central sensitization, since allodynia in late CPIP, but not early CPIP rats, is also relieved by intrathecal treatment with an inhibitor of protein kinase M-zeta, an isoform of protein kinase C that has been shown to be critical for the maintenance of spinal nociceptive plasticity.⁵⁴

While our studies suggest that the topical combinations reduce allodynia by improving microvascular function, we cannot exclude other antiinflammatory or nonspecific effects. In the case of pentoxifylline or lisofylline, these drugs produce multiple antiinflammatory effects including anticytokine, and immune system modulator effects.^{40–42} However, most of the antiinflammatory effects of pentoxifylline and lisofylline stem from their actions to reduce microvascular dysfunction: by reducing the chemotactic signals from damaged endothelial cells that attract immune cells, and by reducing the production of oxygen free radicals and cytokines after improving the oxygenation of tissues affected by poor capillary blood flow. Since we only measured the effects of our treatments on blood flow and oxidative capacity, we cannot speculate on additional antiinflammatory effects. However, additional studies show that our combinations are not effective in late CPIP rats, for which

microvascular dysfunction has resolved (when we have shown here that PORH and oxidative capacity, and previously that muscle perfusion and oxidative capacity are normal).

It could also be argued that both clonidine and apraclonidine produce nonspecific effects at α_1 -adrenoceptors. Like most drugs, the specificity of clonidine and apraclonidine depends on its tissue concentration. For example, evidence suggests that apraclonidine is a relatively selective α_2 -adrenergic agonist, and that its affinity at α_2 -adrenoceptors is 100 to 528-fold greater than its affinity at α_1 -adrenoceptors.⁵⁵ In most tissues, α_2 -adrenoceptors are predominantly pre-junctional receptors producing negative feedback on NE release from sympathetic postganglionic neurons. They may also be present on smooth muscle cells of resistance vessels and cause vasoconstriction, although they are typically extra-junctional and do not normally respond to NE released from sympathetic postganglionic neurons.^{56,57} In some tissues, such as the anterior segment of the eye (conjunctiva, iris, ciliary body), α_2 -adrenergic agonists produce significant vasoconstriction, since there is a much higher proportion of post-junctional vasoconstrictive α_2 -adrenoceptors.⁵⁸ Thus, apraclonidine produces vasoconstriction in the anterior segment of the eye, while producing no vasoactive effects in the posterior segment where post-junctional α_2 -adrenoceptors are less abundant (retina, choroid, and optic nerve).⁵⁹

In conclusion, combining topical drugs designed to enhance local arterial and capillary blood flow to overcome microvascular dysfunction produce effective antiallodynic effects in an animal model of CRPS. We confirmed that one of these combinations reduced microvascular dysfunction, as it increased PORH in CPIP rats. Furthermore, the dramatic enhancement of antiallodynic effects of PA/PDE inhibitors when combined with α_2 A receptor agonists or NO donors, should reduce the potential systemic side effects of these low-concentration topical treatments.

Acknowledgments

Funding: Louise and Alan Edwards Foundation to TJC, Canadian Institutes of Health Research (MOP-143406 to TJC), and Natural Sciences and Engineering Research Council of Canada (194525-09 to TJC)

The authors would like to thank Pfizer Corporation for their generous donation of pregabalin.

References

1. Kurvers HA, Jacobs MJ, Beuk RJ, Van den Wildenberg FA, Kitslaar PJ, Slaaf DW, Reneman RS. Reflex sympathetic dystrophy: evolution of microcirculatory disturbances in time. *Pain*. 1995; 60:333–40. [PubMed: 7596630]
2. Matsumura H, Jimbo Y, Watanabe K. Haemodynamic changes in early phase reflex sympathetic dystrophy. *Scandinavian J Plastic Reconstr Surg and Hand Surg*. 1996; 30:133–8.
3. Schurmann M, Zaspel J, Grandl G, Wipfel A, Christ F. Assessment of the peripheral microcirculation using computer-assisted venous congestion plethysmography in post-traumatic complex regional pain syndrome type I. *J Vasc Res*. 2001; 38:453–61. [PubMed: 11561147]
4. Dayan L, Salman S, Norman D, Vatine JJ, Calif E, Jacob G. Exaggerated vasoconstriction in complex regional pain syndrome-I is associated with impaired resistance artery endothelial function and local vascular reflexes. *J Rheumatol*. 2008; 35:1339–45. [PubMed: 18464300]
5. Koban M, Leis S, Schultze-Mosgau S, Birklein F. Tissue hypoxia in complex regional pain syndrome. *Pain*. 2003; 104:149–57. [PubMed: 12855324]

6. Coderre TJ, Xanthos DN, Francis L, Bennett GJ. Chronic post-ischemia pain (CPIP): a novel animal model of complex regional pain syndrome-type I (CRPS-I; reflex sympathetic dystrophy) produced by prolonged hind paw ischemia and reperfusion in the rat. *Pain*. 2004; 112:94–105. [PubMed: 15494189]
7. Xanthos DN, Bennett GJ, Coderre TJ. Norepinephrine-induced nociception and vasoconstrictor hypersensitivity in rats with chronic post-ischemia pain. *Pain*. 2008; 137:640–51. [PubMed: 18079061]
8. Laferrière A, Millecamps M, Xanthos DN, Xiao WH, Siau C, de Mos M, Sachot C, Ragavendran JV, Huygen FJ, Bennett GJ, Coderre TJ. Cutaneous tactile allodynia associated with microvascular dysfunction in muscle. *Mol Pain*. 2008; 4:49. [PubMed: 18957097]
9. Ragavendran, JV., Laferrière, A., Khorashadi, M., Coderre, TJ. The early anti-allodynic effect of pentoxifylline on chronic post-ischemia pain depends on modulation of plantar blood flow. Abstracts of the XIII World Congress on Pain; 2010; IASP Press; p. PT 186
10. Giessler C, Wangemann T, Silber RE, Dhein S, Brodde OE. Noradrenaline-induced contraction of human saphenous vein and human internal mammary artery: involvement of different alpha-adrenoceptor subtypes. *NS Arch Pharmacol*. 2002; 366:104–9.
11. Hrometz SL, Edelmann SE, McCune DF, Olges JR, Hadley RW, Perez DM, Piascik MT. Expression of multiple alpha1-adrenoceptors on vascular smooth muscle: correlation with the regulation of contraction. *J Pharmacol Exp Ther*. 1999; 290:452–63. [PubMed: 10381812]
12. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*. 1987; 327:524–526. [PubMed: 3495737]
13. Waldman SA, Murad F. Cyclic GMP synthesis and function. *Pharmacol Rev*. 1987; 39:163–196. [PubMed: 2827195]
14. Cubeddu LX. New alpha 1-adrenergic receptor antagonists for the treatment of hypertension: role of vascular alpha receptors in the control of peripheral resistance. *Am Heart J*. 1988; 116:133–162. [PubMed: 2899387]
15. Ignarro LJ, Napoli C, Loscalzo J. Nitric oxide donors and cardiovascular agents modulating the bioactivity of nitric oxide: an overview. *Circ Res*. 2002; 90:21–8. [PubMed: 11786514]
16. Weiss B, Hait WN. Selective cyclic nucleotide phosphodiesterase inhibitors as potential therapeutic agents. *Ann Rev Pharmacol Toxicol*. 1977; 17:441–77. [PubMed: 17360]
17. Rybalkin SD, Beavo JA. Multiplicity within cyclic nucleotide phosphodiesterases. *Biochem Soc Trans*. 1996; 24:1005–9. [PubMed: 8968501]
18. Lincoln TM. Cyclic GMP and mechanisms of vasodilation. *Pharmacol Ther*. 1989; 41:479–502. [PubMed: 2541452]
19. Horlington M, Watson PA. Inhibition of 3'5'-cyclic-AMP phosphodiesterase by some platelet aggregation inhibitors. *Biochem Pharmacol*. 1970; 19:955–6. [PubMed: 4323661]
20. Jacoby D, Mohler ER. Drug treatment of intermittent claudication. *Drugs*. 2004; 64:1657–70. [PubMed: 15257627]
21. Robless P, Mikhailidis DP, Stansby GP. Cilostazol for peripheral arterial disease. *Cochrane Database Syst Rev*. 2007;CD003748. [PubMed: 17253494]
22. Wattanasirichaigoon S, Menconi MJ, Delude RL, Fink MP. Lisofylline ameliorates intestinal mucosal barrier dysfunction caused by ischemia and ischemia/reperfusion. *Shock*. 1999; 11:269–75. [PubMed: 10220304]
23. Rice GC, Brown PA, Nelson RJ, Bianco JA, Singer JW, Bursten S. Protection from endotoxic shock in mice by pharmacologic inhibition of phosphatidic acid. *Proc Natl Acad Sci USA*. 1994; 91:3857–61. [PubMed: 8171002]
24. Davis KD, Treede RD, Raja SN, Meyer RA, Campbell JN. Topical application of clonidine relieves hyperalgesia in patients with sympathetically maintained pain. *Pain*. 1991; 47:309–17. [PubMed: 1664508]
25. Reuben SS, Sklar J. Intravenous regional anesthesia with clonidine in the management of complex regional pain syndrome of the knee. *J Clin Anesth*. 2002; 14:87–91. [PubMed: 11943518]
26. Manahan AP, Burkman KA, Malesker MA, Benecke GW. Clinical observation on the use of topical nitroglycerin in the management of severe shoulder-hand syndrome. *Nebr Med J*. 1993; 78:87–9.

27. Groeneweg G, Niehof S, Wesseldijk F, Huygen FJ, Zijlstra FJ. Vasodilative effect of isosorbide dinitrate ointment in complex regional pain syndrome type 1. *Clin J Pain*. 2008; 24:89–92. [PubMed: 18180642]
28. Cocco G, Strozzi C, Haeusler G, Chu D, Amrein R, Padovan GC. The therapeutic value of clonidine in patients with coronary heart disease. *Eur J Cardiol*. 1979; 10:221–8. [PubMed: 499282]
29. Insel J, Halle AA, Mirvis DM. Efficacy of pentoxifylline in patients with stable angina pectoris. *Angiology*. 1988; 39:514–9. [PubMed: 3377271]
30. Messin R, Fenyvesi T, Carreer-Bruhwyler F, Crommen J, Chiap P, Hubert P, Dubois C, Famaey JP, Géczy J. A pilot double-blind randomized placebo-controlled study of molsidomine 16 mg once-a-day in patients suffering from stable angina pectoris: correlation between efficacy and over time plasma concentrations. *Eur J Clin Pharmacol*. 2003; 59:227–32. [PubMed: 12734607]
31. Sternitzky R, Seige K. Clinical investigation of the effects of pentoxifylline in patients with severe peripheral occlusive vascular disease. *Curr Med Res Opin*. 1985; 9(9):602–10. [PubMed: 4053674]
32. Maksimovi ZV. Prostacyclin and pentoxifylline in the treatment of patients with inoperable occlusions of the peripheral arteries of the lower extremities *Srp. Arh Celok Lek*. 1999; 127:249–53.
33. Kukushkin ML, Smirnova VS, Gorizontova MP, Mironova IV, Zinkevich VA. Effect of clofelin and propranolol on the development of neurogenic pain syndrome in rats. *Patol Fiziol Eksp Ter*. 1993; 4:16–9.
34. Gorizontova MP, Mironova IV. The effect of prophylactic administration of pentoxifylline (trental) on development of a neuropathic pain syndrome and microcirculatory disorders caused by it. *Biull Eksp Biol Med*. 1995; 119:485–7. [PubMed: 7579239]
35. Liu J, Feng X, Yu M, Xie W, Zhao X, Li W, Guan R, Xu J. Pentoxifylline attenuates the development of hyperalgesia in a rat model of neuropathic pain. *Neurosci Lett*. 2007; 412:268–72. [PubMed: 17140731]
36. Ruddock MW, Hirst DG. Pentoxifylline inhibits agonist-induced vasoconstriction in vascular smooth muscle and spontaneous peristalsis in isolated ileum. *Oncol Res*. 2005; 15:81–6. [PubMed: 16119005]
37. Wattanasirichaigoon S, Menconi MJ, Delude RL, Fink MP. Lisofylline ameliorates intestinal mucosal barrier dysfunction caused by ischemia and ischemia/reperfusion. *Shock*. 1999; 11:269–75. [PubMed: 10220304]
38. Magnusson M, Gunnarsson M, Berntorp E, Björkman S, Höglund P. Effects of pentoxifylline and its metabolites on platelet aggregation in whole blood from healthy humans. *Eur J Pharmacol*. 2008; 581:290–5. [PubMed: 18155691]
39. Bhat VB, Madyastha KM. Antioxidant and radical scavenging properties of 8-oxo derivatives of xanthine drugs pentoxifylline and lisofylline. *Biochem Biophys Res Commun*. 2001; 288:1212–7. [PubMed: 11700041]
40. Rice GC, Rosen J, Weeks R, Michnick J, Bursten S, Bianco JA, Singer JW. CT-1501R selectively inhibits induced inflammatory monokines in human whole blood ex vivo. *Shock*. 1994; 1:254–66. [PubMed: 7735959]
41. Waxman K, Daughters K, Aswani S, Rice G. Lisofylline decreases white cell adhesiveness and improves survival after experimental hemorrhagic shock. *Crit Care Med*. 1996; 24:1724–8. [PubMed: 8874313]
42. Du C, Cooper JC, Klaus SJ, Sriram S. Amelioration of CR-EAE with lisofylline: effects on mRNA levels of IL-12 and IFN-gamma in the CNS. *J Neuroimmunol*. 2000; 110:13–9. [PubMed: 11024530]
43. Chen M, Yang Z, Wu R, Nadler JL. Lisofylline, a novel antiinflammatory agent, protects pancreatic beta-cells from proinflammatory cytokine damage by promoting mitochondrial metabolism. *Endocrinology*. 2002; 143:2341–8. [PubMed: 12021199]
44. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods*. 1994; 53:55–63. [PubMed: 7990513]

45. Millecamps M, Coderre TJ. Rats with chronic post-ischemia pain (CPIP) exhibit an analgesic sensitivity profile similar to human patients with complex regional pain syndrome type I (CRPS-I). *Eur J Pharmacol.* 2008; 583:97–102. [PubMed: 18272148]
46. Kumar N, Laferrière A, Yu JSC, Leavitt A, Coderre TJ. Evidence that pregabalin reduces neuropathic pain by inhibiting the spinal release of glutamate. *J Neurochem.* 2010; 113:552–61. [PubMed: 20132471]
47. Stewart WC. Effect and side effects of apraclonidine. *Klin Monatsbl Augenheilkd.* 1996; 209:A7–13.
48. Van Zwieten PA, Thoolen MJ, Timmermans PB. The pharmacology of centrally acting antihypertensive drugs. *Br J Clin Pharmacol.* 1983; 15(Suppl 4):455S–62S.
49. Roustit M, Cracowski JL. Non-invasive assessment of skin microvascular function in humans: an insight into methods. *Microcirculation.* 2012; 19:47–64. [PubMed: 21883640]
50. Creager MA. Clinical assessment of the patient with claudication: the role of the vascular laboratory. *Vasc Med.* 1997; 2:231–7. [PubMed: 9546973]
51. Li C, Sekiyama H, Hayashida M, Takeda K, Sumida T, Sawamura S, Yamada Y, Arita H, Hanaoka K. Effects of topical application of clonidine cream on pain behaviors and spinal Fos protein expression in rat models of neuropathic pain, postoperative pain, and inflammatory pain. *Anesthesiology.* 2007; 107:486–94. [PubMed: 17721252]
52. Groeneweg JG, Huygen FJ, Niehof SP, Wesseldijk F, Bussmann JB, Schasfoort FC, Stronks DL, Zijlstra FJ. No recovery of cold complex regional pain syndrome after transdermal isosorbide dinitrate: a small controlled trial. *J Pain Symptom Manage.* 2009; 38:401–8. [PubMed: 19515530]
53. Groeneweg G, Huygen FJ, Niehof SP, Wesseldijk F, Bussmann JB, Schasfoort FC, Stronks DL, Zijlstra FJ. Effect of tadalafil on blood flow, pain, and function in chronic cold complex regional pain syndrome: a randomized controlled trial. *BMC Musculoskelet Disord.* 2008; 9:143. [PubMed: 18937830]
54. Laferrière A, Pitcher MH, Haldane A, Huang Y, Cornea V, Kumar N, Sacktor TC, Cervero F, Coderre TJ. PKM ζ is essential for spinal plasticity underlying the maintenance of persistent pain. *Mol Pain.* 2011; 7:99. [PubMed: 22185613]
55. Munk SA, Harcourt D, Arasasingham P, Gluchowski C, Wong H, Burke J, Kharlamb A, Manlapaz C, Padillo E, Williams L, Wheeler L, Garst M. Analogs of UK 14304: structural features responsible for α_2 adrenoceptor activity. *Bioorg Med Chem Lett.* 1995; 5:1745–1750.
56. Alabaster V, Davey M. Precapillary vessels: effects of the sympathetic nervous system and of catecholamines. *J Cardiovasc Pharmacol.* 1984; 6 (Suppl 2):S365–76. [PubMed: 6206345]
57. Ruffolo RR Jr. Distribution and function of peripheral alpha-adrenoceptors in the cardiovascular system. *Pharmacol Biochem Behav.* 1985; 22:827–33. [PubMed: 2989947]
58. Wikberg-Matsson A, Simonsen U. Potent alpha(2A)-adrenoceptor-mediated vasoconstriction by brimonidine in porcine ciliary arteries. *Invest Ophthalmol Vis Sci.* 2001; 42:2049–55. [PubMed: 11481271]
59. Costa VP, Harris A, Stefánsson E, Flammer J, Kriegelstein GK, Orzalesi N, Heijl A, Renard JP, Serra LM. The effects of antiglaucoma and systemic medications on ocular blood flow. *Prog Retin Eye Res.* 2003; 22:769–805. [PubMed: 14575724]

Fig. 1.

Sample trace of laser Doppler blood flow (flux) in the hindpaw of an anesthetized rat before (baseline), during (occlusion) and after (PORH) 120 sec of ischemia. Flux levels drop to near zero during the entire ischemic/occlusion period, rapidly increase and gradually reduce to baseline levels during the ~120 sec PORH period.

Fig. 2.

Assessment of the effects of single topical drugs, apraclonidine (A), linsidomine (B), lisofylline (C) and pentoxifylline (D) on paw withdrawal thresholds (PWTs) to von Frey stimulation of the ipsilateral (injured) hindpaw in day 2–14 chronic postischemia pain (CPIP) rats. Singly, each drug produces dose-related antiallodynic effects, with higher concentrations producing significant elevations of PWTs and the lowest concentrations failing to produce significant antiallodynic effects. * $P < 0.05$, ** $P < 0.01$ between pre- and post-drug mean PWTs.

Fig. 3.

Assessment of the effects of topical combinations of lisofylline given with either vehicle or ineffective concentrations of apraclonidine (A,B), or pentoxifylline given with either vehicle or ineffective concentrations of linsidomine (C,D) on paw withdrawal thresholds (PWTs; A,C) and antiallodynic (Δ PWT) dose-response curves (B,D) in the ipsilateral (injured) hindpaw of day 2–14 chronic postischemia pain (CPIP) rats. The combinations significantly increased PWTs at concentrations much lower than those in Fig. 2, and shifted the antiallodynic dose-response curve for lisofylline or pentoxifylline between 5 and 10 fold to the left. * $P < 0.05$ between pre- and post-drug mean PWTs.

Fig. 4.

Comparison of the effects of topical vehicle application, or ipsilateral versus contralateral application of the most effective concentrations of apraclonidine + lisofylline (A) or linsidomine + pentoxifylline (B), on paw withdrawal thresholds (PWTs) in the ipsilateral (injured) hindpaw of day 2–14 chronic postischemia pain (CPIP) rats. In all trials, topical application of vehicle failed to significantly alter PWTs. Furthermore, although each combination significantly increased PWTs when applied to the ipsilateral hindpaw, they were all ineffective when applied to the contralateral hindpaw. $*P < 0.05$ between pre- and post-drug mean PWTs; $†P < 0.05$ from post-drug vehicle; $‡P < 0.05$ from post-drug contralateral PWT.

Fig. 5.

Assessment of the time course of topical combinations of apraclonidine + lisofylline (A) or linsidomine + lisofylline (B) on paw withdrawal thresholds (PWTs) to von Frey stimulation of the ipsilateral (injured) hindpaw in day 2–14 chronic postischemia pain (CPIP) rats. The combinations produced antiallodynic effects lasting 3–6 h after the topical application (* $P < 0.05$, ** $P < 0.01$ compared to pre-drug baseline (0 h)).

Fig. 6.

A) Assessment of the effects of either systemic pregabalin (10 mg/kg, i.p.), low dose topical application of apraclonidine and lisofylline, or a combination of systemic pregabalin and the topical combination on paw withdrawal thresholds (PWTs) to von Frey stimulation of the ipsilateral (injured) hindpaw in day 2–14 (early) chronic postischemia pain (CPIP) rats.

While 10 mg/kg systemic pregabalin produced no effect on its own, when combined with the topical combination of apraclonidine + lisofylline, it produced antiallodynic effects that were significantly greater than the topical combination alone (* $P < 0.05$, ** $P < 0.01$ compared to pre-drug; † $P < 0.05$ compared to post-drug topical combination, ¶ $P < 0.05$ compared to post-drug vehicle-treated animals).

B) Assessment of the effects of either systemic pregabalin (10 mg/kg, i.p.), low dose topical application of apraclonidine and lisofylline, or a combination of systemic pregabalin and the topical combination on paw-withdrawal thresholds (PWTs) to von Frey stimulation of the ipsilateral (injured) hind paw in day 18 – 26 (late) CPIP rats. While pregabalin was effective on its own at 10 mg/kg, the addition of topical apraclonidine + lisofylline did not lead to greater antiallodynic effects (* $P < 0.05$, ** $P < 0.01$ compared to pre-drug; † $P < 0.05$ compared to post-drug topical combination, ¶ $P < 0.05$ compared to post-drug vehicle).

Fig. 7.

Time course of laser Doppler blood flow (flux % baseline) changes in the 120 s post-occlusive reactive hyperemia (PORH) period in day 5–10 chronic postischemia pain (CPIP) rats treated with vehicle (A) or apraclonidine + lisofylline (B) or in sham rats treated with vehicle (C) or apraclonidine + lisofylline (D). Pre-drug flux during PORH was lower in CPIP rats (A, B) compared to shams (C, D), and flux during PORH was significantly increased post-drug relative to pre-drug only in CPIP rats treated with apraclonidine + lisofylline (B) (* $P < 0.05$, ** $P < 0.01$ versus pre-drug measures taken at the same time of PORH).

Fig. 8.

Areas under the curve of laser Doppler flux (AUCs) during the s post-occlusive reactive hypermia (PORH) period for chronic postischemia pain (CPIP) or sham rats treated with vehicle or apraclonidine + lisofylline both pre- (white bars) and post- (black bars) drug. Pre-drug AUCs for CPIP rats are significantly lower than sham rats (* $P < 0.05$; ** $P < 0.01$), and for CPIP rats treated with apraclonidine + lisofylline the post-drug AUC is significantly higher than the pre-drug AUC (** $P < 0.01$).

Fig. 9.

Formazan levels (scaled to total sample protein) measured in hindpaw plantar skin (A–D) and plantar muscle (E–H) from early (A,B,E,F) or late (C,D,G,H) chronic postischemia pain (CPIP) or sham rats treated with vehicle or apraclonidine + lisofylline both pre (white bars) and post- (black bars) drug. Formazan levels are significantly lower in plantar skin and muscle from the ipsilateral ischemia-reperfusion (I/R)-injured side of vehicle-treated early CPIP animals compared to the contralateral (uninjured) side (* $P < 0.05$, ** $P < 0.01$), and in muscle compared to the vehicle- and the drug-treated side of sham rats (‡ $P < 0.05$ and ¶ $P < 0.01$ respectively). For both muscle and skin, there is also a significant

difference between pre- and post drug levels in early CPIP animals ($\dagger P < 0.05$). None of these effects were found in late CPIP and sham animals.