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Muscle Inflammation Induces a Protein Kinase Cε–Dependent Chronic-Latent Muscle Pain

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Abstract: **Skeletal muscle injuries can induce chronic pain, but the underlying mechanism is unknown. One possible cause has been suggested to be an increased sensitivity to inflammatory mediators. We demonstrate that self-limited inflammatory hyperalgesia induced by intramuscular carrageenan (lasting ~5 days) results in a state of chronic-latent hyperalgesia, revealed by injection of prostaglandin E_2 (PGE_2) 10 days after carrageenan at the same site. In carrageenan-pretreated muscle, PGE_2 produced hyperalgesia that was unattenuated even 14 days after injection, markedly longer than the 4-hour hyperalgesia induced by PGE_2 in naive rats. This chronic-latent hyperalgesia was reversed as well as prevented by spinal intrathecal injection of oligodeoxynucleotide antisense to protein kinase Cε, a second messenger implicated in long-lasting plasticity in cutaneous nociceptors.**

Perspective: ***We describe a novel experimental model for chronic muscle pain, produced by mild acute muscle inflammation, that has clinical significance since it has the potential to reveal cellular processes by which acute inflammation or muscle trauma underlies chronic muscle pain.***

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Key words: *Muscle pain, PKC epsilon, prostaglandin E2, carrageenan, hyperalgesia.*

Chronic muscle pain is a constellation of symptoms that develops after trauma or in association with repetitive strain. It is believed to be dependent, at least in part, on muscle inflammation,[6,38,39] because it responds to nonsteroidal anti-inflammatory drugs[19] and cytokine levels are increased in the symptomatic muscle.[21,33] Although symptoms may improve over time, they return with the use of the involved muscle, even years later.[15,20,35] Unfortunately, little is known about the cellular mechanisms underlying chronic muscle pain,[8] in particular the mechanism mediating the transition from acute to chronic pain.

Recently, we established a model of chronic-latent inflammatory pain in cutaneous tissues. In this model, a single exposure to the inflammogen carrageenan (a classic agent for the induction of experimental inflammation and inflammatory pain that is relevant to clinical inflammatory pain states[9,10,14]) produced a prolonged hypersensitivity to subsequent exposure of hyperalgesic agents.[4,12,27,28] In this "hyperalgesic priming," or chronic-latent hyperalgesia, the inflammatory mediator prostaglandin (PG) E_2 and other inflammatory agents that act directly on nociceptors (eg, 5-hydroxtryptamine and adenosine) produced an enhanced and markedly prolonged hyperalgesia (>24 hours compared with <4 hours in naive rats) when injected several weeks after the initial response to carrageenan has resolved, a plastic change in nociceptor function mediated by PKCε. Since chronic muscle pain is believed to be dependent, at least in part, on muscle inflammation,[6,38,39] we investigated whether chronic hyperalgesia develops in muscle after recovery from transient inflammation.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (weight, 250–400 g) were housed in the Animal Care Facility at the University of California San Francisco (UCSF), under environmentally controlled conditions (7 AM–7 PM light cycles; 21°–23°C) with food and water available ad libitum. Care and use of rats conformed to National Institutes of Health

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guidelines and was approved by the UCSF Institutional Animal Care and Use Committee. Effort was made to minimize the number of animals used and their suffering.

Measurement of Hyperalgesia

Mechanical nociceptive thresholds were quantified using a digital force transducer (Chatillon DFI2; Amtek Inc., Largo, FL) with a custom-made 6-mm-diameter probe attached to the transducer. Rats were lightly restrained in a plexiglas holder that allows for easy access to the hind limb and application of the transducer probe to the belly of the gastrocnemius muscle. The nociceptive threshold was defined as the force, in Newtons, required to produce a flexion reflex in the hind leg. Baseline withdrawal threshold was defined as the mean of 3 readings taken at 5-minute intervals. Each hind limb was treated as an independent measure and each experiment was performed on a separate group of rats. Animals were followed over time. All behavioral testing was done between 10 am and 4 pm.

Intramuscular Injections

Rats were briefly anesthetized with 3% isoflurane, and vehicle (0.9% saline) or drug was injected into the belly of the gastrocnemius muscle in 1 hind limb (in a volume of 10 μL); the skin over the injection sites was marked with indelible pen so that the same area could be repeatedly tested for mechanical nociceptive threshold.

Carrageenan Hyperalgesia

Carrageenan (100 μg in 10 μL 0.9% saline) was injected into the belly of the gastrocnemius muscle. In our previous studies on carrageenan-induced nociceptive priming in cutaneous tissue, we administered 50 μg in 5 μL,[4,28] that is, half the dose used in the current muscle nociceptive priming study. The 100 μg/10 μL dose of carrageenan was determined in pilot studies as sufficient to produce robust muscle primary mechanical hyperalgesia, equivalent to that seen in the cutaneous model with 50 μg/5 μL. It is likely that this dose/volume difference is due to the greater volume of distribution at the muscle injection site compared with intradermal injections.

Antisense Oligodeoxynucleotide Preparation

Since priming develops over a period of several days, continuous inhibition of PKCε by administration of antagonists (which have a relatively short half-life) into muscle would be technically challenging. Therefore, to address the role of PKCε in muscle afferents, we used antisense oligonucleotides to PKCε to reduce expression of this enzyme, thereby producing a functional block of neuronal PKCε activity.

The 20-mer PKCε antisense oligodeoxynucleotide (ODN) sequence, 5′-GCC AGC TCG ATC TTG CGC CC-3′, was directed against a unique sequence of rat PKCε. The corresponding GenBank (National Institutes of Health, Bethesda, MD) accession number and ODN position within the cDNA sequence are XM345631 and 226-245, respectively. The mismatch ODN sequence, 5′-GCC AGC **G**CG ATC TT**T** CGC CC-3′, corresponds to the PKCε subunit antisense sequence with 2 bases mismatched (in bold typeface). We have previously shown that this antisense ODN against PKCε decreases PKCε protein in dorsal root ganglia.[11]

For intrathecal injection of ODN, rats were briefly anesthetized with 3% isoflurane; a 30-guage needle was inserted into the subarachnoid space on the midline between the L4 and L5 vertebrae, and ODN (80 μg in 10 μL) was slowly injected. Control animals received injections of mismatch ODN.[1-3,13,17,18,26-28]

Experimental Groups

Experiment 1: Carrageenan Hyperalgesia

We tested the effects of carrageenan on nociceptive threshold injection by measuring basal nociceptive mechanical threshold of the gastrocnemius muscle, then administering a single injection of carrageenan (100 μg in 10 μL, n = 3 to 6), and then testing mechanical nociceptive threshold at 30, 60, and 120 minutes and 1, 2, 3, 4, and 7 days after injecting carrageenan. A control group received intramuscular injection of 0.9% saline (10 μL) instead of the carrageenan (n = 3 to 6).

Experiment 2: Chronic Latent Hyperalgesia

To determine whether a prior administration of carrageenan could induce a state of chronic-latent hyperalgesia, a single carrageenan injection (100 μg in 10 μL, n = 3 to 6) was administered to the gastrocnemius muscle. Ten days after this injection, nociceptive thresholds were assessed to confirm that they had returned to precarrageenan injection levels; the inflammatory mediator PGE_2 (1 μg) was then injected into the gastrocnemius muscle. Nociceptive threshold was then reassessed at 1 and 4 hours and again 1, 2, and 7 days after PGE_2 administration.

Experiment 3: PKCε Dependence

To determine whether PKCε contributes to hyperalgesic priming in muscle, we first tested whether attenuating PKCε before exposure to carrageenan could prevent hyperalgesic priming. PKCε ODN (80 μg/20 μL, n = 6) was administered intrathecally in 1 group of rats once daily for 3 days before carrageenan and then daily for 5 days after carrageenan. Ten days after carrageenan injection, nociceptive thresholds were assessed (they had returned to precarrageenan injection levels) and the inflammatory mediator PGE_2 (1 μg) was injected into the gastrocnemius muscle. Nociceptive threshold was then reassessed at 1 and 4 hours and again 1, 3, 9, and 14 days after PGE_2 administration. To determine whether attenuating PKCε can reverse hyperalgesia priming, in a different group of rats PKCε ODN was administered intrathecally once daily for 3 days beginning 5 days after carrageenan (to test reversal). Nociceptive thresholds were determined before carrageenan administration and again 2 and 5 days after injection (to determine

acute carrageenan hyperalgesia and recovery of nociceptive threshold to precarrageenan levels). Eight days after carrageenan injection, PGE_2 (1 μg) was injected into the gastrocnemius muscle and nociceptive threshold was assessed at 1 hour, 4 hours, and 1 and 4 days after PGE_2 administration.

Statistics

Group data are expressed as mean ± SEM of n observations in hind limbs. Statistical comparisons were made by using repeated-measures ANOVA, with Bonferroni post hoc test, using StatView statistical software (SAS Institute, Inc., Cary, NC).

Results

Carrageenan Hyperalgesia

The mechanical threshold to elicit leg withdrawal decreased by ~60% within 2 hours of the intramuscular injection of carrageenan (100 μg) and remained at approximately this level for at least 2 days (saline, open circles, versus carrageenan, filled circles; $P < .0001$, repeated-measures ANOVA; Fig 1). By day 4, nociceptive thresholds had returned to baseline. Control animals injected with 0.9% saline vehicle (open circles) exhibited no significant change in nociceptive threshold.

Chronic-Latent Hyperalgesia

After complete recovery from the acute hyperalgesia induced by intramuscular carrageenan (which occurs ~4 days after carrageenan administration; Fig 1), the response to a new inflammatory challenge was assessed. Ten days after carrageenan administration, after verifying the return to precarrageenan baseline withdrawal threshold, the inflammatory mediator PGE_2 (1 μg) was injected into the gastrocnemius muscle (time 0). In saline-pretreated control animals (open circles), PGE_2 induced a short-lived hyperalgesia that completely resolved within 4 hours. In contrast, in the carrageenan-primed muscle (filled circles), the duration of PGE_2-induced hyperalgesia was significantly and markedly prolonged, remaining undiminished for at least 1 week after PGE_2 administration (2-way ANOVA, $P < .001$; Fig 2).

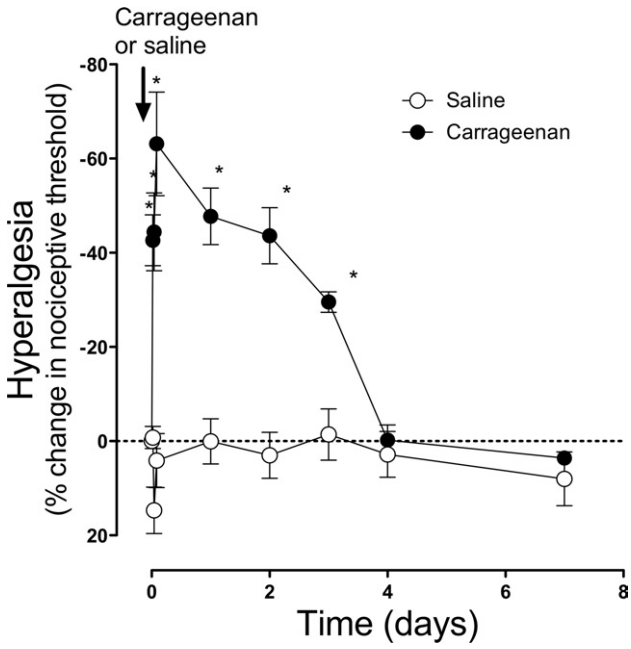


Figure 1. Carrageenan muscle hyperalgesia. Carrageenan (1%; 30 μL, filled circles, n = 3–6) injected into the gastrocnemius muscle of adult male rats decreased nociceptive threshold by ~60% within 1 day, and nociceptive threshold was lower than in saline-injected muscle for 3 days. By day 4, nociceptive threshold was not significantly different from baseline (and saline-treated) values. Control animals injected with saline (0.9% NaCl, 30 μL, open circles, n = 3–6) exhibited no significant change in threshold.

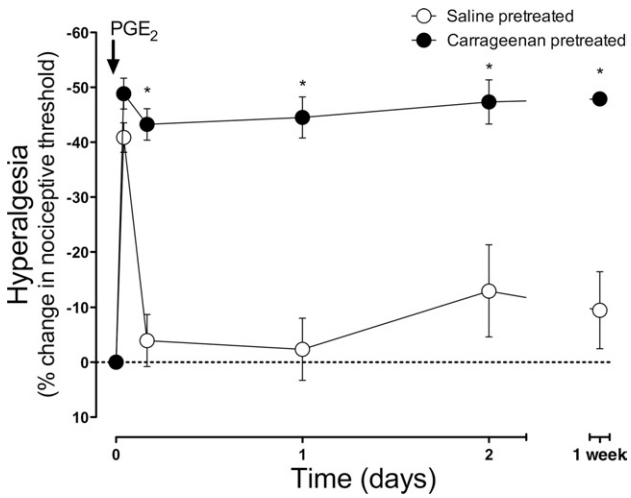


Figure 2. Chronic-latent muscle hyperalgesia. Ten days after carrageenan (1%; 30 μL), PGE_2 (100 ng) was injected into the muscle. In saline-pretreated rats (open circles, n = 3–6), PGE_2-induced hyperalgesia had completely resolved within 3 hours, but in the carrageenan-pretreated muscle (filled circles, n = 6), the duration of hyperalgesia was greatly enhanced, remaining undiminished 1 week after PGE_2 admininstration.

PKCε Dependence

To determine whether PKCε contributes to hyperalgesic priming in muscle, as it does in skin,[4] we determined if PKCε ODN could prevent the development of and/or reverse chronic-latent hyperalgesia. PKCε ODN (80 μg/20 μL) was administered intrathecally once daily for 3 days before carrageenan and then daily for 5 days after carrageenan (to test prevention) or daily for 3 days beginning 5 days after carrageenan (to test reversal). PGE_2 hyperalgesia was evaluated 6 days after the last PKCε ODN administration (ie, 11 days after carrageenan) to test for prevention or 1 day after the last PKCε ODN administration (ie, 8 days after carrageenan) to test for reversal. PKCε ODN (filled circles, n = 6) treatment completely prevented the development of carrageenan chronic-latent hyperalgesia (mismatch versus antisense, $P < .0001$, repeated-measures ANOVA; Fig 3) while not affecting the magnitude or duration of the acute phase of PGE_2 hyperalgesia. PKCε ODN (filled circles, n = 6) treatment also reversed hyperalgesic priming when injected after recovery from carrageenan hyperalgesia

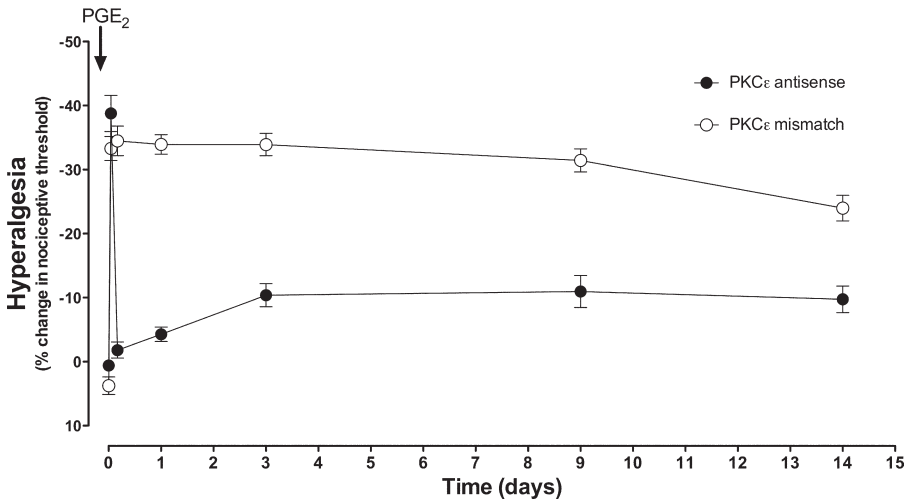


Figure 3. PKCε antisense prevents chronic-latent hyperalgesia. Three days before and 5 days after intramuscular carrageenan (1%; 30 μL in gastrocnemius), PKCε mismatch ODN (open circles, n = 6) or antisense (filled circles, n = 6), was injected intrathecally once daily. Six days after the final mismatch ODN, PGE_2 (100 ng, injected into the gastrocnemius) produced a decrease in nociceptive threshold that lasted at least 14 days. In contrast, PGE_2 hyperalgesia in PKCε antisense ODN-treated rats (filled circles, n = 6) was significantly shorter and similar in duration to that in saline-pretreated rats (compare with open circles, Fig 2).

(mismatch versus antisense, $P < .0001$, repeated-measures ANOVA; Fig 4).

Discussion

Chronic muscle pain is a major health problem,[22,40] due in part to its long-term persistence and in part to its recurrence after resolution of acute symptoms, after resumption of precipitating activity (eg, musicians or athletes resuming their occupation[15,20,35]). This clinical picture suggests that in these patients there exists a latent hyperalgesic state that may be unmasked after an innocuous triggering event (eg, minor mechanical insult) to produce moderate to severe pain. In the current study we have demonstrated that intramuscular carrageenan induces a state of chronic-latent mechanical hyperalgesia. This state can be demonstrated, after recovery from acute carrageenan-induced muscle hyperalgesia (which lasts ~5 days), when PGE_2 is injected at the same intramuscular site to produce a markedly prolonged hyperalgesic response that lasts at least 2 days, compared with ~3 hours in control animals. We have previously described a similar phenomenon in cutaneous nociceptors,

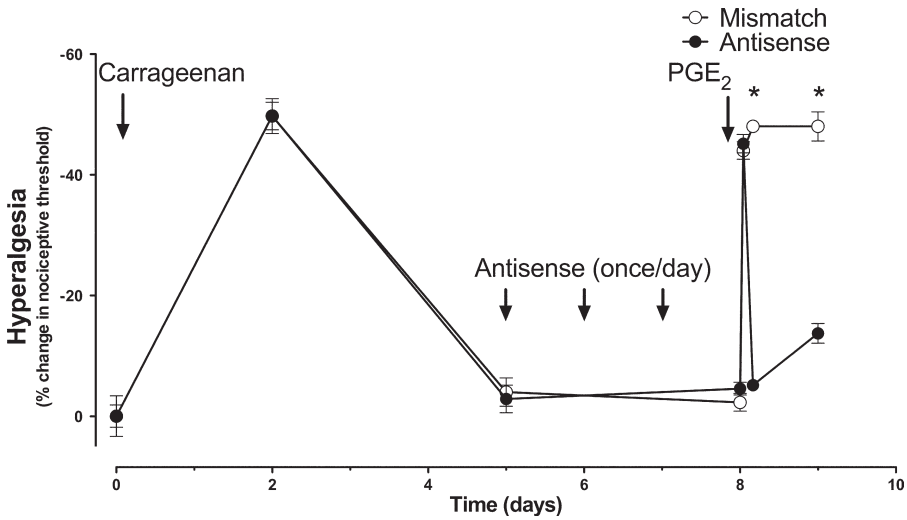


Figure 4. PKCε antisense reverses chronic-latent hyperalgesia. PKCε mismatch ODN (open circles, n = 6) or antisense (filled circles, n = 6) was injected intrathecally daily for 3 days, 5 days after intramuscular carrageenan (1%; 30 μL in gastrocnemius) when nociceptive thresholds had returned to baseline. One day after the final ODN injection, PGE_2 (100 ng, injected into the gastrocnemius) was injected into the muscle. In PKCε antisense ODN- injected rats (filled circles, n = 6), PGE_2 hyperalgesia was present 1 hour after PGE_2, but by 4 hours nociceptive threshold had returned to baseline, similar to the effect in saline pretreated rats (see open circles in Fig 2). In contrast, in PKCε mismatch ODN-administered rats, nociceptive threshold was significantly decreased even 24 hours after PGE_2 administration.

a phenomenon that we have termed hyperalgesic priming. An important feature of hyperalgesic priming is a switch in the second messenger for inflammatory mediator–induced hyperalgesia. Thus, whereas PGE_2 hyperalgesia in the skin is normally PKC-independent, during hyperalgesic priming, PGE_2 hyperalgesia is now mediated by the novel PKC isoform PKCε.[4,28] The development of hyperalgesic priming can be prevented by attenuation of PKCε in the primary afferent nociceptor using spinal intrathecal administration of ODN antisense to PKCε. Similar to what we observed in the skin, chronic-latent hyperalgesia in muscle was prevented after pretreatment with PKCε antisense. We also observed that hyperalgesic priming could be reversed when PKCε antisense is administered after carrageenan administration.

Although we cannot exclude a contribution from central/spinal sites to chronic-latent muscle hyperalgesia, we have previously shown that intrathecally administered PKCε ODN antisense significantly reduces PKCε expression in peripheral dorsal root ganglion neurons. Furthermore, in rats treated with PKCε ODN antisense, a specific peptide inhibitor of PKCε (εV1-2 peptide) that attenuates peripherally mediated hyperalgesia induced by epinephrine in naive or PKCε ODN mismatch-treated rats was no longer effective.[28] Thus, although it is possible that central nociceptive circuitry could also be affected by PKCε, the available data indicate a critical role for PKCε in peripheral nerve endings.

After recovery from a short-lived hyperalgesia produced by 100 μg carrageenan, we observed a chronic-latent hyperalgesia characterized by a dramatically prolonged PGE_2-induced hyperalgesia. Previous studies have shown that carrageenan induces muscle hyperalgesia when given at much higher doses,[16,21,31] but at these doses administration of carrageenan into rat gastrocnemius muscle produces myonecrosis (at 3 mg)[31] and myositis (at 15 mg).[7,34] It did not produce mechanical hyperalgesia when given at 300 μg.[31] Our ability to detect acute carrageenan mechanical hyperalgesia at a much lower dose (100 μg) may be dependent on our use of a 6-mm-diameter probe, which is likely to activate many more muscle C-fiber afferents than the von Frey hairs (diameter ~1 mm) used in previous studies.

There have been several attempts to develop appropriate animal models for muscle pain, for example, by intramuscular injection of inflammatory agents such as carrageenan (4 mg),[16] capsaicin,[36] or formalin.[33] However, at the doses used in these studies, these agents produce overt tissue damage and immune cell infiltration,[33] whereas other models using intramuscular injection of TNF-α or acidic saline have been shown to produce muscle pain without causing damage to the muscle tissue or immune cell recruitment.[33,37] However, these approaches have been criticized as not adequately modeling tonic or persistent types of muscle pain syndromes,[32] and acidic saline produces a bilateral hyperalgesia after a unilateral injection, suggesting a central sensitization, contrasting with clinical muscle pain after injury or in myofascial pain syndromes, wherein pain tends to be localized to specific muscles rather than a more generalized pain syndrome.[23] Our animal model of chronic muscle pain, using very low-dose carrageenan as the initiating inflammatory stimulus, should be useful for future studies designed to determine the underlying mechanisms of chronic muscle pain.

Clinically, one of the most important aspects of inflammatory pain is the development of chronic pain after acute inflammation, for example, as produced in repetitive strain disorders.[24] We have hypothesized that this process involves cellular mechanisms different from those of acute inflammation and that after the resolution of a transient inflammatory event, a long-lasting state of enhanced responsiveness to subsequent hyperalgesic stimuli can exist. In this study, we describe a novel experimental model for muscle pain produced by mild acute muscle inflammation. This model has clinical significance because it tracks the transition from acute to chronic-latent peripheral muscle hyperalgesia and has the potential to reveal cellular processes by which acute inflammation or muscle trauma can create a state of enhanced susceptibility to inflammatory mediators or subsequent mechanical stimulation. These findings have begun to clarify mechanisms underlying chronic muscle pain; this model has the potential to provide information for future strategies for the prevention and treatment of chronic musculoskeletal pain. Of particular importance in this regard is whether muscle pain resulting from eccentric activity, repetitive ergonomic strain, and/or vibration (all of which are believed to involve an inflammatory component[5,25,29,30]) also produces a state of chronic-latent hyperalgesia. We are currently developing models of these work-related musculoskeletal pain syndromes to test this hypothesis.

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