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Minocycline inhibits the enhancement of antidromic primary afferent stimulation-evoked vasodilation following intradermal capsaicin injection

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Abstract

Neurogenic inflammation is induced by inflammatory mediators released in peripheral tissue from primary afferent nociceptors. Our previous studies suggest that neurogenic inflammation induced by intradermal injection of capsaicin results from the enhancement of dorsal root reflexes (DRRs), which involve antidromic activation of dorsal root ganglion (DRG) neurons. Numerous studies have reported the important role of glial modulation in pain. However, it remains unclear whether glial cells participate in the process of neurogenic inflammation-induced pain. Here we tested the role of DRG satellite glial cells (SGCs) in this process in anesthetized rats by administration of a glial inhibitor, minocycline. Electrical stimuli (ES, frequency 10 Hz; duration 1 ms; strength 3 mA) were applied to the cut distal ends of the $L_{4\text{-}5}$ dorsal roots. The stimuli evoked antidromic action potentials designed to mimic DRRs. Local cutaneous blood flow in the hindpaw was measured using a Doppler flow meter. Antidromic ES for 10 min evoked a significant vasodilation that could be inhibited dose-dependently by local administration of the calcitonin gene-related peptide receptor antagonist, $CGRP_{8\text{-}37}$. Pretreatment with capsaicin intradermally injected into the hindpaw 2 h before the ES enhanced greatly the vasodilation evoked by antidromic ES, and this enhancement could be reversed by minocycline pretreatment. Our findings support the view that neurogenic inflammation following capsaicin injection involves antidromic activation of DRG neurons via the generation of DRRs. Inhibition of neurogenic inflammation by minocycline is suggested to be associated with its inhibitory effect on SGCs that are possibly activated following capsaicin injection.

Keywords

neurogenic inflammation; capsaicin; minocycline; satellite glial cells

Neurogenic inflammation is produced by overstimulation of primary afferent nociceptive terminals due to injury or inflammation of peripheral tissues [35]. Upon stimulation, a variety of inflammatory mediators, such as neuropeptides, are released from terminals of these nociceptors [21,28]. Neuropeptides, including calcitonin gene-related peptide (CGRP)

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and substance P (SP), are synthesized and stored in medium- and small-sized primary afferent nociceptive neurons with lightly myelinated Aδ and unmyelinated C axonal fibers in dorsal root ganglia (DRG). It has been accepted by our and other groups that the release of neuropeptides is driven by antidromic stimulation of primary afferent nociceptive fibers, which is mainly mediated by dorsal root reflexes (DRRs) [46]. In an animal model of neurogenic inflammation induced by intradermal injection of capsaicin (CAP), our group has demonstrated that neurogenic inflammation and the resulting sensitization of primary afferent nociceptors evoked by CAP injection result from antidromic activation of DRG neurons [26,28]. In this process, the expression of CGRP in DRG neurons is increased [48].

Recent findings have highlighted the active role of glia in the pathogenesis of pain. Peripheral painful stimulation induces activation of spinal microglial cells [3,7,34,41], in which a cross-talk between glial cells and sensory neurons linked by pro-inflammatory agents, such as cytokines, released from activated microglial cells contribute critically to the development and maintenance of pain [32,42]. In sensory ganglia, activated satellite glial cells (SGCs) are found to play a major role in neuropathic pain [43]. However, whether SGCs are involved in neurogenic inflammation remains unclear. Experiments in this study were designed to test if neurogenic inflammation (vasodilation) induced by antidromic activation of DRG neurons involves the activation of SGCs. Some preliminary data have been published in abstract form [10,27].

Experiments were conducted on male Sprague-Dawley rats, weighing 250–350 g. They were housed two per cage, with free access to food and water, in the animal facility center with a 12-h alternating light-dark cycle. All experimental protocols were approved by the Animal Care and Use Committee of the University of Texas at Arlington and were in accordance with the guidelines of the National Institutes of Health and the International Association for the Study of Pain.

Rats were initially anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The external jugular vein was cannulated, and anesthesia was maintained by continuous infusion of sodium pentobarbital (5–8 mg/kg/h). Anesthesia was kept at a sufficient level as judged by the absence of corneal reflexes and withdrawal reflexes. Rectal temperature was monitored using a rectal probe and maintained at 37°C by a servo-controlled heating blanket.

Cutaneous blood flow in the hindpaw was detected as blood cell flux by a laser Doppler flowmeter (Moor Instruments, UK) and vasodilation (a major characteristic of inflammation) in response to antidromic activation of primary afferents was evaluated by measuring changes in blood flow using a laser Doppler flow meter probe attached to the skin of the foot, as described previously [28,29]. The output showing blood flow level was then recorded by a computer data acquisition system (CED 1401^{+}, with Spike-2 software) in mV units [28] (also see Figs. 1 and 2).

In order to perform antidromic electrical stimulation (ES) of primary afferents, the L_4 and L_5 dorsal roots were exposed by laminectomy and their identity was confirmed by their relation to the corresponding vertebra. Dorsal rhizotomy was performed on the ipsilateral side where the blood flow in the hindpaw was measured, as described before [28]. A bipolar silver electrode was placed on the cut distal ends of dorsal rootlets. Warm mineral oil pools contained by skin flaps kept the nerves from drying and cooling. The parameters for antidromic ES were 10 Hz, 1 ms, and 3 mA, which have been shown to activate C-fibers [4,15].

Close-by intra-arterial injection was used to deliver a CGRP receptor antagonist, $CGRP_{8\text{-}37}$, to the periphery [26]. To do this, one branch of the femoral artery on the side ipsilateral to the blood flow measurement was carefully isolated from connective tissue and ligated

proximally. The artery was then cannulated distally by a small-sized polyethylene tube that was connected with a Hamilton syringe. $CGRP_{8-37}$ (from TOCRIS), dissolved in saline at doses of 10 or 40 μg [26], was injected intra-arterially in a volume of 25 μl at 5 min before antidromic ES. For control purposes, saline (25 μl) was given using the same procedure.

One percent CAP (from Sigma/Aldrich, 20 μl, prepared in a solution of 7% Tween 80 and 93% saline) was injected intradermally (i.d.) into the plantar surface of the foot after dorsal rhizotomy [29]. The vehicle (7% Tween 80 and 93% saline) was injected i.d. as a control, which has been shown in our previous study to produce no obvious changes in blood flow [29].

Experiments on the effects of glial cell inhibition were conducted on rats that had undergone a pretreatment with minocycline, a glial inhibitor (from Sigma/Aldrich), that was injected intraperitoneally (i.p.) at a dose of 50 mg/kg daily for 7 days. The administration schedule we used in the study was based on our preliminary studies in which glial inhibitor pretreatment could inhibit neurogenic inflammation and the resulting sensitization of primary afferent nociceptors [10,27]. For control purposes, saline used for dissolving minocycline was administered using the same procedure.

All values are presented as means±SEM. Baseline blood flow level (pre-antidromic ES) was expressed as 100% and percentage changes after antidromic ES were compared for groups of animals that received different treatments. One-way ANOVA was used to compare the difference among the groups with different treatments, and values were considered to be significantly different when $P < 0.05$. All statistical calculations were performed with a statistical software package (SPSS, Version 13.0).

When ES were applied antidromically to the cut distal ends of L_4 and L_5 dorsal roots, an immediate increase in cutaneous blood flow was evoked in the hindpaw skin ipsilateral to the ES side (Fig. 1A). The increased blood flow lasted for about 10 min and then recovered gradually even though the stimuli were continuously given (Fig. 1A). Thus, 10-min of ES was chosen to test the responses of blood flow to antidromic activation of primary afferents. Changes in the blood flow in the forepaw skin during antidromic ES of the cut distal ends of dorsal roots were monitored simultaneously, and no changes were found (Fig. 1B), which means that the evoked blood flow changes in the hindpaw were site and stimulation specific, not the result of a stimulation-induced systemic effect.

To see if vasodilation evoked by antidromic activation of primary afferents was mediated by the release of neuropeptides, such as CGRP, intra-arterial administration of the CGRP receptor antagonist, $CGRP_{8-37,}$ was applied to the hindpaw ipsilateral to the ES at 5 min prior to antidromic ES. We found that $CGRP_{8-37}$ pretreatment could inhibit the increase in blood flow evoked by antidromic ES in a dose-dependent manner. The peak increase was significantly reduced from 272.6±34.8% (saline pre-treated, n=7) to 228.7±27.4% after 10 μg $CGRP_{8-37}$ was administered as a pretreatment ($p<0.05$, compared with saline pre-treated, n=7), and to 181.2±13.3% ($p<0.01$, compared with saline pretreatment, n=6) after 40 μg $CGRP_{8-37}$ was given (Fig. 1C and D).

In the following experiments, we wanted to test further if antidromic ES-evoked vasodilation is affected by intradermal injection of CAP and effects of pretreatment with minocycline. Blood flow responses to antidromic ES without minocycline pretreatment and CAP injection [vehicle (**Veh**) treatments] were tested to serve as controls for minocycline (**Mino**) and/or CAP treatments. Consistent with the results shown in Fig. 1A, antidromic ES evoked a significant vasodilation in the rats without minocycline and CAP treatments (**i.p. Veh+i.d. Veh**, in Fig. 2A and the first bar in 2E), and the peak increase was 229.4±38.9% (n=8, the first bar in Fig. 2E). In the group with minocycline pretreatment (**i.p. Mino+i.d.**

Veh, n=8), antidromic ES induced an increase in blood flow for 202.6±38.9% (Fig. 2B and the second bar in 2E). However, there was no statistically significant difference between these two groups (p=0.966, Fig. 2E). The above experiments were further performed under the condition where the hindpaw was injected intradermally with CAP at 2 h before the experiments. Vasodilation was produced following CAP injection, and returned nearly completely to the control level 2 h after injection (data not shown), which was consistent with our previous observations [29]. Interestingly, 2 h after CAP injection, the vasodilation evoked by antidromic ES was greatly enhanced (Fig. 2C). The test was made after saline pretreatment, and the peak increase was 543.8±59.5% (the third bar in Fig. 2E, $p<0.01$, compared with saline injection). Importantly, the enhancement of evoked vasodilation was profoundly inhibited after pretreatment with minocycline (Fig. 2D and the fourth bar in Fig. 2E). The peak increase dropped to 121.0±23.8% (n=8, $p<0.01$, compared with the saline pretreatment group having CAP injection).

The main findings of the present study are that vasodilation in the hindpaw evoked by antidromic ES can be enhanced by intradermal injection of CAP. This enhancement is significantly prevented by minocycline pretreatment that inhibits glial cells. Thus, activation of SGCs in DRG is suggested to make an important contribution to the CAP-induced neurogenic inflammation.

Antidromic primary afferent stimulation-evoked inflammatory responses are characterized by vasodilation and/or edema. These responses are stimulus strength dependent and most significant when the strength is sufficient to activate C-fibers [19,20,39]. In the current study, a significant vasodilation was evoked by antidromic ES of dorsal roots using the parameters of ES similar to those used by other groups [4,19,36]. It is well accepted that inflammatory responses evoked by antidromic activation of primary nociceptive afferents are due to the release of pro-inflammatory agents, such as CGRP and/or SP, in the target tissue [21,22,24,25,37]. Antidromic activation of primary afferents was presumed to mimic the vasodilation induced by triggering of DRRs [8,28,30]. Data obtained from our and other groups and the current study demonstrate that neurogenic inflammation evoked by antidromic activation of primary afferent nociceptive fibers is partially explained by CGRP release because a blockade of peripheral CGRP receptors inhibited dose-dependently the evoked vasodilation [8,28].

The current study has also shown that the antidromic ES-evoked vasodilation could be significantly enhanced by CAP injection. Since the enhancement was seen around 2 h after CAP injection, it does not seem to be due to a direct effect on the peripheral endings of primary afferents. Many studies have shown that plastic changes in DRG neurons occur after peripheral injury, inflammation or persistent nociceptive stimulation, which is due to up-regulation of nociceptive molecules, such as the transient receptor potential vanilloid-1 ($TRPV_1$) receptors, and/or CGRP and SP, in the neurons [16,18,33,48]. Studies by our group using a rat model of intradermal CAP injection suggest strongly that up-regulation of $TRPV_1$ receptors and hyperactive synthesis of CGRP in DRG neurons plays a critical role in DRR-mediated neurogenic inflammation and the resulting sensitization of primary afferent nociceptors [27,28,47,48]. Therefore, plastic changes in DRG nociceptive neurons should contribute to the mechanism of DRR-mediated neurogenic inflammation.

Studies on pain models, particularly the neuropathic pain models, have indicated that sensitization of sensory neurons in responses to peripheral injury, inflammation or persistent nociceptive stimulation is closely associated with activation of microglial cells in the spinal cord and SGCs in DRG [6,14,44]. It has been well documented that neuron-glia signaling promotes hyperexcitability of sensory neurons by the release of a variety of mediators, such as pro-inflammatory cytokines, between neurons and glia. For example, adenosine 5′-

triphosphate released from the cell bodies of DRG neurons can activate the surrounding SGCs [50]. CAP injection leads to an enhancement of neuronal-satellite glial signaling in trigeminal ganglia [5,9,40]. Upon activation, glial cells synthesize a plethora of compounds, such as tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), and cyclooxygenase [32,34,45,49]; these have been widely reported to cause hyperexcitablity of sensory neurons [1,11,12].

In the present study, we found that the CAP-induced enhancement of neurogenic inflammation could be dramatically inhibited by pretreatment with minocycline, a glial inhibitor. However, minocycline treatment did not produce any effect on vasodilation evoked by antidromic stimulation if CAP was not injected. Minocycline is a broad-spectrum antibiotic that inhibit microglial activation and proliferation [13,31,32,38]. The effects of minocycline have been suggested to involve multiple mechanisms of action specifically on glial cells, which include suppression of microglial phosphorylated-p38 mitogen-activated protein kinase (MAPK) [2,17] and down-regulation of microglial production of pro-inflammatory cytokines, such as IL-1β and TNF-α [23], and prostaglandin E2 [51]. It has been demonstrated that minocycline can alleviate pain through the above mechanisms without evidence of direct effects on neurons either at spinal or DRG levels [17,31]. It has been reported that preemptive and repeated injection of minocycline can produce a cumulative drug effect that is more effective than acute administration in inhibition of activated glial cells [2,31]. Thus, it is strongly indicated in the present study that there is activation of glial cells following CAP injection, which participates in the process of neurogenic inflammation. Based on the experimental manipulation of dorsal rhizotomy in which dorsal roots at lumbosacral level have been sectioned, we favor the opinion that the glia activated and involved in neurogenic inflammation are mainly the SGCs in the DRG. Direct evidence remains to be provided in future plan by anatomically investigating satellite glial activation following CAP injection and its contribution to neurogenic inflammation.

In summary, this study supports the view that neurogenic inflammation following CAP injection involves antidromic activation of DRG neurons via the generation of DRRs. Importantly, minocycline inhibition of neurogenic inflammation is likely associated with its inhibitory effect on SGCs that are possibly activated following CAP injection. Thus, we propose that this should be an important component of the pathological mechanisms of neurogenic inflammation.

Research Highlights

- Cutaneous vasodilation evoked by antidromic electrical stimulation of the primary afferents can be enhanced by intradermal injection of capsaicin.
- Capsaicin induced enhancement is significantly prevented by chronic minocycline pretreatment that inhibits glial cells.
- Inhibition of neurogenic inflammation by minocycline is suggested to be associated with its inhibitory effect on satellite glial cells that are possibly activated following capsaicin injection.

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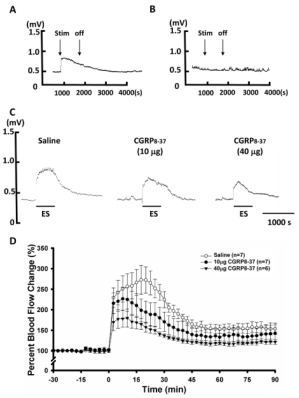

Figure 1.

Increase in cutaneous blood flow in the hindpaw evoked by antidromic electrical stimulus (ES) of L4 and L5 dorsal roots and the effects of blockade of peripheral CGRP receptors by intra-arterial injection of $CGRP_{8-37}$. **A:** Sample of the laser Doppler flowmeter trace that shows the increase in blood flow in the hindpaw evoked by antidromic ES. **B:** Sample of the trace that shows no change in blood flow in the forepaw when L4 and L5 dorsal roots were antidromically stimulated. **C:** Samples of the traces showing the effects of blockade of CGRP receptors on the antidromic ES evoked vasodilation by pretreatment of the periphery with two different doses of $CGRP_{8-37}$. Saline pretreatment using the vehicle for dissolving $CGRP_{8-37}$ served as a control (left panel). Horizontal lines below traces show times of application of the ES. **D:** Grouped data summarizing that $CGRP_{8-37}$ inhibited dose-dependently the increase in blood flow evoked by antidromic ES.

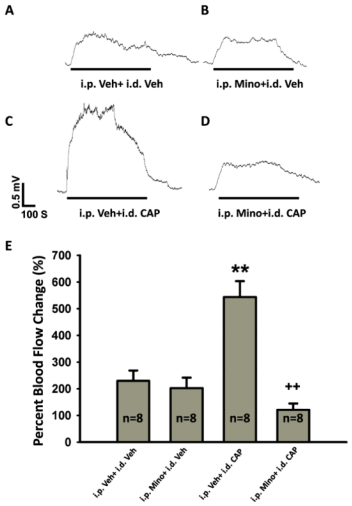

Figure 2.

Enhancement of the antidromic ES-evoked vasodilation following CAP injection and the effects of glial cell inhibition by pretreatment with minocycline. **A:** Antidromic ES-evoked vasodilation without minocycline and CAP treatments [treatments with vehicle (Veh) for minocycline (Mino) and CAP treatments]. **B:** Antidromic ES-evoked vasodilation with minocycline and without CAP treatment. **C:** Antidromic ES-evoked vasodilation without minocycline and with CAP treatment. **D:** Antidromic ES-evoked vasodilation with minocycline and CAP treatments. Horizontal lines below traces show times of application of the ES. **E**: Grouped data summarizing the changes in antidromic ES-evoked vasodilation following CAP injection and the effects of minocycline treatment. ** $p<0.01$, compared with the group without minocycline and CAP treatments (**i.p. Veh+i.d. Veh**). ++ $P<0.01$, compared with the group without minocycline treatment (**i.p. Veh+i.d. CAP**).