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Phytochemical quercetin alleviates hyperexcitability of trigeminal nociceptive neurons associated with inflammatory hyperalgesia comparable to NSAIDs

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Abstract

Quercetin is a flavonoid that is widely found in fruits and vegetables. Quercetin inhibits cyclooxygenase-2 and modulates voltage-gated ion channels, however, its effect on nociceptive neuron-associated inflammatory hyperalgesia remains unknown. The present study investigated under in vivo conditions whether systemic administration of quercetin attenuates the inflammation-induced hyperexcitability of trigeminal spinal nucleus caudalis (SpVc) neurons associated with mechanical hyperalgesia and compared its effect to the non-steroidal anti-inflammatory drug, diclofenac. Complete Freund's adjuvant was injected into the whisker pads of rats to induce inflammation, and then mechanical stimulation was applied to the orofacial area to assess the threshold of escape. The mechanical threshold was significantly lower in inflamed rats compared to uninjected naïve rats, and this lowered threshold returned to control levels 2 days after administration of quercetin or diclofenac. The mean discharge frequency of SpVc wide-dynamic range (WDR) neurons to both non-noxious and noxious mechanical stimuli in inflamed rats was significantly decreased after quercetin or diclofenac administration under combination of three anesthetic agents (medetomidine, midazolam and butorphanol). In addition, the increased mean spontaneous discharge of SpVc WDR neurons in inflamed rats significantly decreased after quercetin or diclofenac administration. Similarly, quercetin or diclofenac restored the expanded mean receptive field size in inflamed rats to control levels. In this study, the combination of three anesthetic agents did not result in any obvious "noxious pinch-evoked after discharges" in CFA inflamed day 2 rat as described previously in pentobarbital-anesthetized rats. Together, these results suggest that administration of quercetin attenuates inflammatory hyperalgesia associated with hyperexcitability of nociceptive SpVc WDR neurons *via* inhibition of the peripheral cyclooxygenase-2 signaling cascade and voltage-gated ion channels. These findings support the proposed potential of quercetin as a therapeutic agent in complementary alternative medicine strategies for preventing trigeminal inflammatory mechanical hyperalgesia.

Keywords

Inflammation, trigeminal nociceptive neuron, hyperalgesia, single-unit recording, cyclooxygenase-2, diclofenac, quercetin

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Introduction

For orofacial sensory processing, the spinal trigeminal nucleus caudalis (SpVc) is an important relay station for neural trigeminal nociceptive inputs following inflammation and tissue injury.[1,2] SpVc nociceptive neurons are classified as nociceptive-specific or wide-dynamic range (WDR) based on their sensitivity to mechanical stimulation applied to orofacial

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areas, such as facial skin, with WDR neurons responsive to both noxious and non-noxious stimulation.[2] Since graded noxious stimuli applied to receptive fields results in increased firing frequency of SpVc WDR neurons in proportion to stimulus intensity, it can be assumed that WDR neurons are important for encoding stimulus intensity. Chronic pathological conditions, such as tissue inflammation can change the properties of somatic sensory pathways, leading to hyperalgesia.[3] Specifically, inflammation and tissue injury change the excitability of primary afferent neurons (peripheral sensitization), which alters information processing in the trigeminal spinal nucleus or higher centers (central sensitization).[4] Complete Freund's adjuvant (CFA) models of inflammation in the orofacial region have been developed in rats to study the trigeminal neural signaling pathways underlying pathological pain,[5–8] with CFA inflammation-induced hyperexcitability of SpVc WDR neurons linked to mechanical stimuli.[5,7] SpVc neurons have also been implicated in the mechanism of hyperalgesia and/or referred pain associated with dental pain.[2,7–8]

Recent reports have described the use of complementary and alternative medicines (CAMs), such as herbal medicines and acupuncture, for the treatment of persistent clinical chronic pain,[9] supporting the potential of CAMs in preventing trigeminal inflammatory hyperalgesia. Chronic administration of dietary constituents, including polyphenols, polyunsaturated fatty acids, and carotenoids, has been shown to attenuate inflammation-induced mechanical hyperalgesia, primarily by suppressing SpVc WDR neuronal hyperexcitability via both peripheral and central cyclooxygenase (Cox)-2 cascade signaling pathways.[10–13] Quercetin is one of the most common flavonoids, a plant metabolite found in dietary phytochemicals present in the daily diet of humans.[14] These phytochemicals have a variety of biological functions, with antioxidant, anti-inflammatory and cardioprotective effects.[15–17] A modulatory role has been reported for quercetin on voltage-gated Na (Nav), K (Kv) and Ca^{2+} (Cav) channels in cardiac muscle.[18,19] In addition, quercetin can also decrease the production of prostaglandin E_2 (PGE_2) by inhibiting Cox-2 cascades.[20–22] The known toxic side effects associated with the most commonly prescribed analgesic drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs) and Cox-2 inhibitors, has increased the interest in CAMs for the treatment of persistent chronic pain.[23,24] Diclofenac is a proven, commonly prescribed NSAID that has analgesic, anti-inflammatory, and antipyretic properties, and has been shown to be effective in treating a variety of acute and chronic pain and inflammatory conditions.[25] Together, these observations strongly suggest that quercetin administration attenuates inflammation-induced hyperexcitability of the SpVc WDR neurons associated with trigeminal hyperalgesia, and could represent a potential therapeutic agent for preventing inflammatory hyperalgesia. To our knowledge, no studies have investigated this proposal. Therefore, using behavioral and electrophysiological techniques, the present study investigated whether quercetin administration under in vivo conditions attenuates inflammation-induced hyperexcitability of the SpVc neurons associated with hyperalgesia in rats. In addition, we also examined and compared the potency of suppression of hyperalgesia-associated, inflammation-induced SpVc neuronal excitability with quercetin and NSAIDs, such as diclofenac.

Materials and methods

All experiments reported herein were approved by the Animal Use and Care Committee of Azabu University and were performed in accordance with the ethical guidelines of the International Association for the Study of Pain.[26] Every effort was made to minimize the number of animals used and their suffering.

Induction of inflammation and administration of quercetin and NSAIDs

The experiments were performed on adult male Wistar rats (body weight 210–260 g, $n = 24$). Rats were divided into four groups, as follows; naïve ($n = 6$), inflamed ($n = 6$), inflamed rats with quercetin (50 mg/kg, i.p.; Sigma-Aldrich, Milano, Italy) treatment ($n = 6$) and inflamed rats with diclofenac (50 mg/kg, i.p.; Sigma-Aldrich, Milano, Italy) treatment ($n = 6$). Previous studies indicated these doses of quercetin and diclofenac treatment significantly suppress Cox-2 activity in vitro.[20–22,27,28] Each animal was anesthetized with 3% isoflurane, and then CFA (0.05 mL 1:1 oil/saline suspension) was injected into the left side of the facial skin, as described previously.[10–13] For naïve rats, vehicle only (0.9% NaCl) was injected into the left side of the facial skin. Quercetin and diclofenac were dissolved in dimethyl sulfoxide (DMSO) and administered chronically to the rats over 2 days. Behavioral experiments were conducted immediately prior to daily administration. Based on the behavioral analysis for escape threshold, electrophysiological experiments were conducted only on day 2 in the inflamed group. In some experiments, we also tested systemic administration of vehicle (DMSO) on day 2 in the inflamed group.

Mechanical threshold for escape behavior

Mechanical threshold for escape behavior was conducted as described previously.[10] In brief, from one to 3 days after CFA or vehicle injection into the facial skin, the ipsilateral and contralateral skin regions were tested to assess mechanical hyperalgesia using a set of von Frey hairs (Semmes-Weinstein Monofilaments, North Coast Medical, CA). To evaluate the rat's escape threshold, the von Frey mechanical stimuli were applied to the whisker pad in an ascending series of trials. Each von Frey stimulation was applied three times in each series of trials. Escape threshold intensity was

determined when rats moved their heads away from at least one of the three stimuli.

Extracellular single-unit recording of SpVc WDR neuronal activity

Electrophysiological recordings were conducted 2 days after CFA or vehicle injection as described previously.[7] Electrophysiological recordings were made in 24 adult male Wistar rats. Each rat was anesthetized with 3% isoflurane and maintained with additional doses of a combination of anesthetic (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam and 5.0 mg/kg of butorphanol) at 2–3 mg/kg/h as required, through a cannula into the jugular vein. Single-neuron activity was recorded through a glass micropipette filled with 2% pontamine sky blue and 0.5 M sodium acetate and/or tungsten microelectrodes (impedance 3MΩ), and recording location was determined by stereotaxic coordinates. Neuronal activity was amplified (WPI, DAM 80), filtered (0.3–10 KHz), and monitored with an oscilloscope (Iwatsu, SS-7672, Tokyo) for off-line analysis by Power Lab and Chart 5 software (ADInstruments, Oxford, UK).

Experimental protocols

The analyses of extracellular single-unit SpVc WDR activity responding to mechanical stimulation of the whisker pad were conducted as follows. To avoid sensitization of peripheral mechanoreceptors, a paint brush was quickly used as a search stimulus to identify the approximate area of receptive field in the left side of the whisker pad. Next, the left side of the whisker pad was searched for single units that responded to a set of von Frey hairs with non-noxious (0.2, 0.6, 2, 6, 10 g) and noxious (15, 26, 60 g) mechanical stimulation for 5 s at intervals of 5 s (Takeda et al., 2012). We identified the criterion for WDR neurons as graded non-noxious and noxious mechanical stimulation applied to the receptive field produces increased firing frequency in proportion to stimulus intensity. After identification of nociceptive SpVc WDR neurons responding to the whisker pad, we determined the threshold for mechanical stimulation, and the size of the receptive field. The mechanical receptive field of neurons was mapped by probing the facial skin with von Frey hairs, and then outlined on a life–sized drawing of the rat on tracing paper.[7,29] WDR neuronal discharges induced by mechanical stimulation were quantified by subtracting the background activity from the evoked activity. Spontaneous discharge frequencies were determined over 2–5 min. Since previous studies have demonstrated that WDR neurons in the SpVc region have an important role in the mechanism underlying hyperalgesia and referred pain associated with orofacial pain,[7] the focus of the present study was on the effects of quercetin on nociceptive SpVc WDR neuronal activity, but we did not examine nociceptive-specific neurons**.** Peristimulus histograms (bin = 100 ms) were generated in response to each stimulus. Afterdischarges were recorded for 10 s after pinching the receptive field. Mean spontaneous and mechanical stimulation-evoked discharge frequencies, afterdischarge frequencies, and mean mechanical thresholds of SpVc WDR neurons were compared among the four animal groups (naïve, CFA, CFA rat with quercetin treatment and CFA rat with diclofenac treatment).

Identification of recording site

At the end of recording sessions, anodal DC currents (30 μA, 3 min) were passed through a recording micropipette before the animals were transcardially perfused with saline and 10% formalin. Frozen coronal sections were cut into 30-μm sections and stained with hematoxylin-eosin. Recording sites were identified as blue spots and electrode tracks were constructed by combining with micromanipulator readings, as described previously.[7]

Data analysis

Values are expressed as means ± SEM. Statistical analysis was performed using one-way repeated measure analysis of variances followed by Tukey-Kramer tests (*post hoc* test) for behavioral and electrophysiological data. A p value <0.05 was considered statistically significant.

Results

Inflammation-induced hyperalgesia

In this study, after CFA injection into the whisker pad, the rats were tested for hyperalgesia by probing the injected site and/or the orofacial skin with von Frey filaments. As shown in Figure 1 in inflamed rats, CFA significantly reduced the threshold for escape from mechanical stimulation applied to the whisker pad area from 54.3 ± 5.7 g in naïve rats to 2.5 ± 1.2 g at day 2 after the injection ($n = 6$, $p < 0.05$; Figure 1). No significant changes in the contralateral threshold in the whisker pad area were observed between the two groups (naïve vs. inflamed; 68.1 ± 3.8 g vs. 59.3 ± 4.2 g, $n = 8$, not significant [NS]).

Chronic administration of quercetin or diclofenac for hyperalgesia

Following daily quercetin administration, the reduced escape threshold from mechanical stimulation in day 1 inflamed rats was partially returned to control levels, but still at a significantly lower level compared with naïve rats (Figure 1). As shown in Figure 1, the reduced escape threshold from mechanical stimulation in inflamed rats returned to control levels following administration of quercetin at day 2 after inflammation (naive vs. day 2 inflamed with quercetin; 53.5 ± 12.3 g vs. 41.2 ± 8.6 g, $n = 6$, NS). Also, the

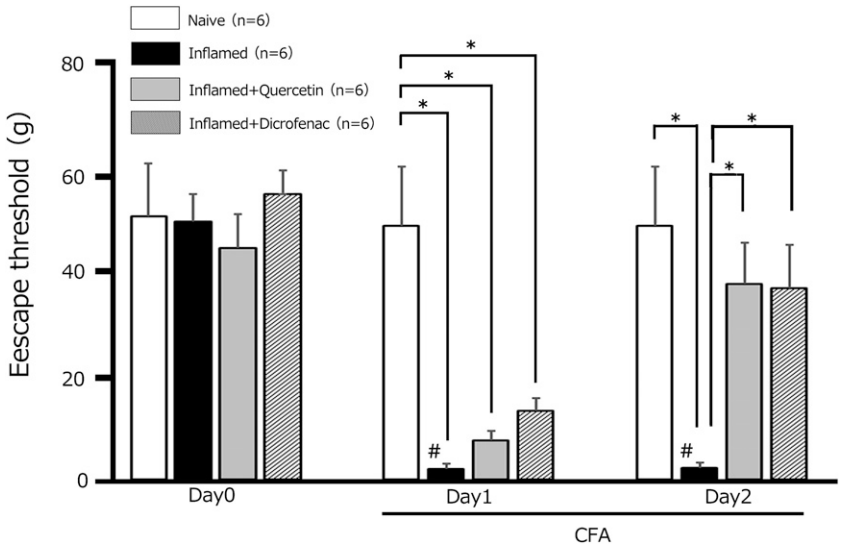

Figure 1. Comparison of changes in the escape threshold among naïve, inflamed, and inflamed with quercetin or diclofenac rats. Mechanical stimulation using von Frey hairs was applied to the ipsilateral whisker pad of naïve (saline; $n = 6$), complete Freund's adjuvant (CFA)-inflamed ($n = 6$), and CFA-inflamed with quercetin (50 mg/kg, i.p.; $n = 6$) or diclofenac (50 mg/kg, i.p.; $n = 6$) rats to assess hyperalgesia. Data are mean ± SEM; # represents $p < 0.05$ when comparing inflamed day 0 vs. inflamed day 1, day 2. * represents $p<0.05$ comparing naive vs. inflamed rats and inflamed vs. inflamed with quercetin or diclofenac.

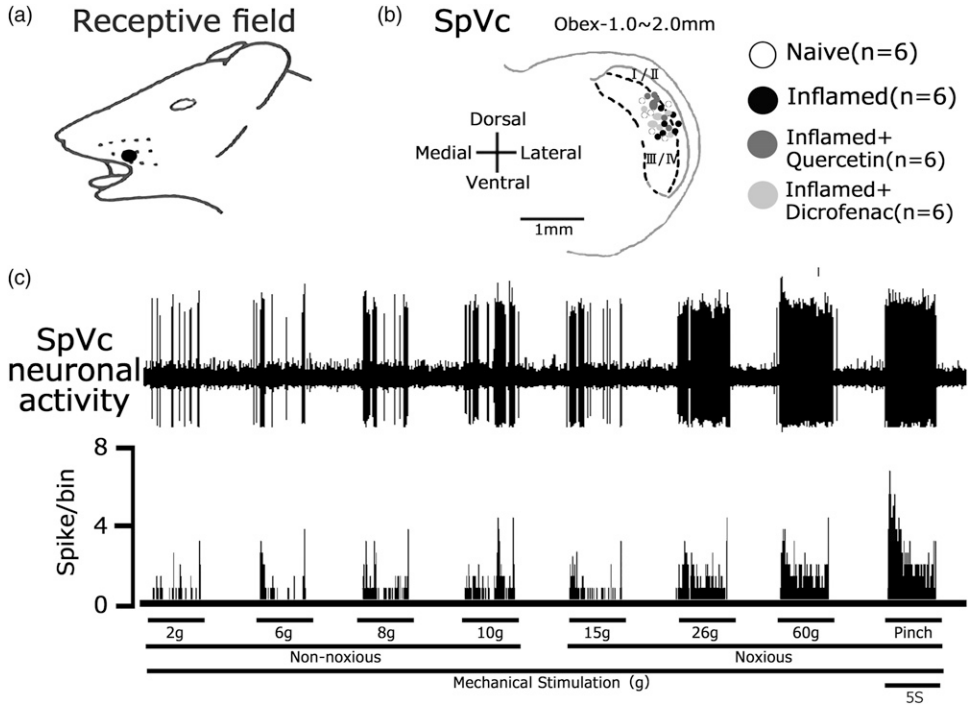

Figure 2. General characteristics of spinal trigeminal nucleus caudalis (SpVc) wide-dynamic range (WDR) neuronal activity in orofacial skin. (A) Receptive field of whisker pad in the facial skin. **(B)** Distribution of SpVc WDR neurons responding to non-noxious and noxious mechanical stimulation of facial skin ($n = 24$). **(C)** Example of non-noxious and noxious mechanical stimulation-induced firing of SpVc WDR neurons.

reduced escape threshold from mechanical stimulation in inflamed rats returned to control levels following administration of diclofenac at day 2 after inflammation (naive vs. day 2 inflamed with quercetin; 53.5 ± 12.3 g vs. 40.3 ± 9.1 g, $n = 6$, NS). There was no significant difference between inflamed rats administered with quercetin or diclofenac at day 2.

Changes in excitability of SpVc WDR neurons following inflammation

A total of 24 SpVc WDR neurons responded to mechanical stimulation of the whisker pad in rats across the naïve, inflamed, inflamed + quercetin and inflamed + diclofenac groups. As shown in Figure 2(a), SpVc neurons responding to non-noxious and noxious mechanical stimulation exhibited a somatic receptive field in the whisker pad area. The recording sites (layers I-II, $n = 5$, 21%; layers III-V, $n = 19$, 79%) were typically distributed in the maxillary branch (Figure 2(b)) with no obvious differences across recording sites among the three groups. In every analyzed SpVc neuron, graded mechanical stimulation applied to the most sensitive area of the receptive field showed increased firing frequency proportional to stimulus intensity. Therefore, every neuron analyzed belonged to the category of WDR neurons (Figure 2(c)), as described in our previous study.[10]

We first confirmed that CFA induced hyperexcitability of SpVc WDR neurons, as described in our previous studies.[10–13] In naïve rats, spontaneous discharges were observed in 17% (1/6) of SpVc neurons (Figure 3(a) and Figure 4(c)) while most neurons fired at a low frequency with a mean firing frequency of 0.1 ± 0.2 Hz ($n = 6$). In contrast, all WDR neurons (6/6; 1.9 ± 0.6 Hz) were spontaneously active in inflamed rats (Figure 3(b) and Figure 4(c)). SpVc WDR neurons in inflamed rats showed significantly stronger responses to non-noxious mechanical stimulation compared with naïve rats (Figure 3(b) and Figure 4(a)), as described previously (Sekiguchi et al., 2016). The mean firing frequencies of SpVc WDR neurons in response to mechanical stimuli (0.4, 2, 15, 60 g, pinch) were also significantly greater in inflamed rats than in control rats ($n = 6$; Figure 4(a)), and the mean mechanical threshold in inflamed rats was significantly decreased to 0.4 ± 0.1 g compared with 2.5 ± 0.5 g in naïve rats ($n = 6$; Figure 4(b)). The mean spontaneous discharge frequency of inflamed rats was significantly increased compared to that of naïve rats (Figure 4(c)). The mean

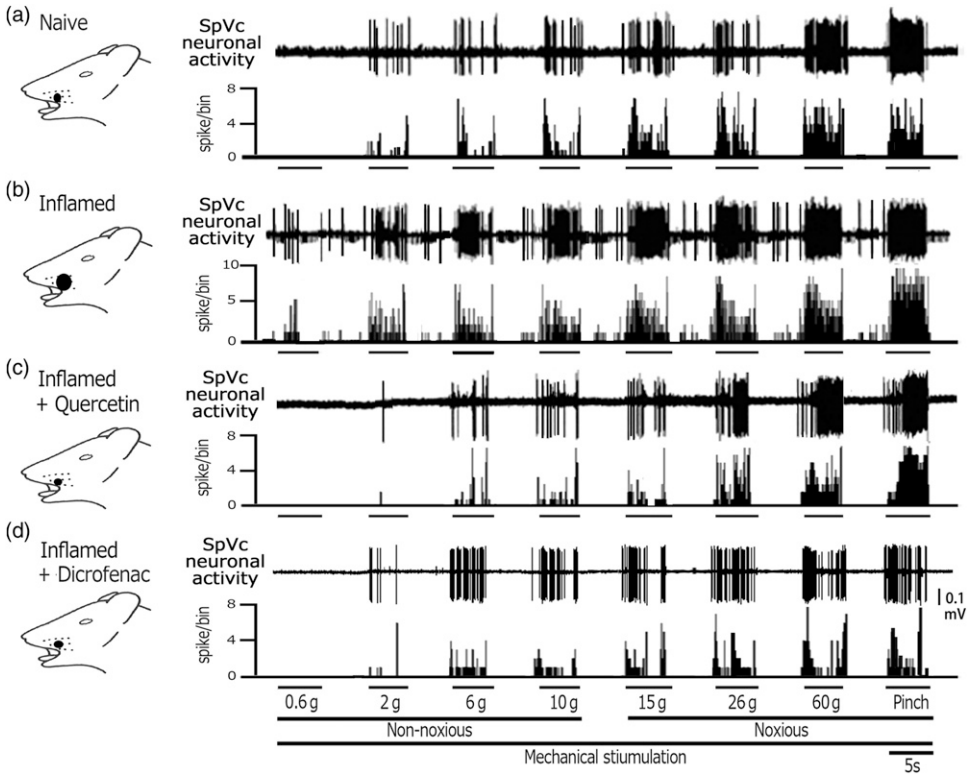

Figure 3. Chronic quercetin or diclofenac administration reverses the hyperexcitability of SpVc WDR neuronal activity after orofacial CFA inflammation. Example of non-noxious and noxious mechanical stimulation-induced discharge of SpVc WDR neurons in naïve ($n = 6$), inflamed ($n = 6$), and inflamed with quercetin (50 mg/kg, i.p. for 2 days; $n = 6$) or diclofenac (50 mg/kg, i.p. for 2 days; $n = 6$) rats. Note the decreased mechanical stimulation threshold required to evoke neuronal firing, increased spontaneous discharges and increased receptive field size in inflamed rats returned to control levels following quercetin or diclofenac administration for 2 days.

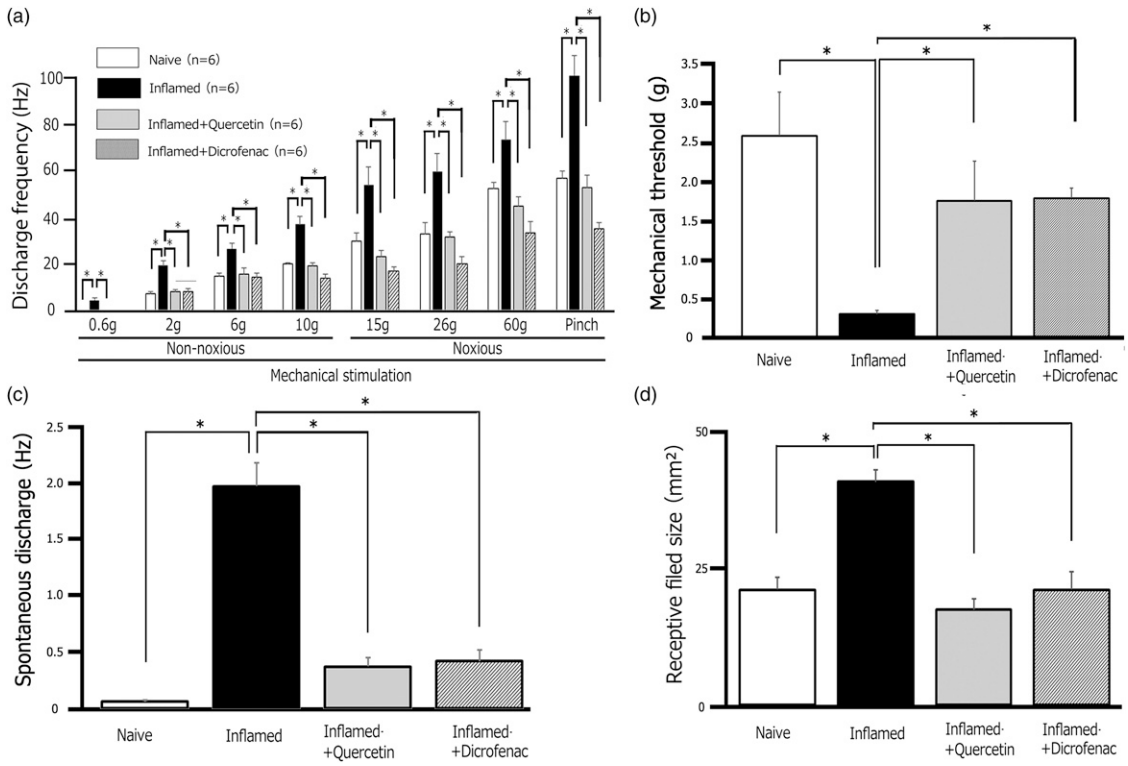

Figure 4. Summary of chronic quercetin or diclofenac administration reverses the hyperactivity of SpVc WDR neuronal activity after orofacial CFA inflammation. (A) Comparison of mean discharge frequency of SpVc WDR neurons evoked by mechanical stimulation (non-noxious and noxious) of orofacial skin among the four rat groups. *represents p <0 .05 comparing naive vs. inflamed rats and inflamed vs. inflamed with quercetin or diclofenac. **(B)** Comparison of mean mechanical threshold of SpVc WDR neurons among the four rat groups. * represents p <0 .05 comparing naive vs. inflamed rats and inflamed vs. inflamed with quercetin or diclofenac **(C)** Spontaneous discharge of SpVc WDR neurons among the four rat groups. *represents $p < 0.05$ comparing naive vs. inflamed rats and inflamed vs. inflamed with quercetin or diclofenac. **(D)** Comparison of mean receptive field size of SpVc WDR neurons among the four rat groups. *represents $p < .05$ comparing naive vs. inflamed rats and inflamed vs. inflamed with quercetin or diclofenac.

receptive size in the inflamed rats was significantly increased to 41.0 ± 2.2 mm^2 compared to 21.2 ± 2.7 mm^2 ($n = 7$, $p <$ 0.05; Figure 4(d)).

In this study, the combination of three anesthetic agents (medetomidine, midazolam and butorphanol), did not result in any obvious "noxious pinch-evoked after discharges" in CFA inflamed day 2 rat (0/6, 0%) as described previously in pentobarbital-anesthetized rats.[10-13]

Chronic administration of quercetin inhibits hyperexcitability of SpVc WDR neurons in inflamed rats

Using a behavioral analysis for escape threshold, we tested the effect of chronic administration of quercetin on the hyperexcitability of SpVc WDR neurons in inflamed day 2 rats. Representative examples of discharge rates for SpVc WDR neurons responding to non-noxious (0.6–10 g) and noxious mechanical (15–60 g, pinch) stimulation following quercetin administration in inflamed rats are shown in Figure 3(c). After daily administration of quercetin for 2 days in inflamed rats, the discharge frequency of SpVc WDR neurons to both non-noxious and noxious mechanical stimulation decreased to control levels (Figure 3(c)). The lowered mechanical threshold, and augmented spontaneous and noxious and non-noxious firing frequencies in inflamed rats returned to that observed in naïve rats. As shown in Figure 4(a), the mean discharge frequency of SpVc WDR neurons in inflamed rats significantly decreased after quercetin administration with both non-noxious and noxious mechanical stimuli ($p < 0.05$). The mean mechanical stimulation threshold in inflamed rats after quercetin was also significantly reversed to control levels (Figure 4(b)). Spontaneous discharge of SpVc WDR neurons in inflamed rats also significantly decreased after quercetin administration (Figure 4(c), $p < 0.05$). The mean receptive field size in inflamed rats significantly decreased to control levels (Figure 4(d)). Chronic vehicle (DMSO) administration had no significant effect on spontaneous and non-noxious, noxious mechanical or pinch stimulation-evoked hyperexcitability of SpVc WDR neurons in inflamed rats (data not shown).

Chronic administration of diclofenac inhibits hyperexcitability of SpVc WDR neurons in inflamed rats

We then tested the effect of chronic administration of diclofenac on the hyperexcitability of SpVc WDR neurons in inflamed day 2 rats. Representative examples of discharge rates for SpVc WDR neurons responding to non-noxious (0.6–10 g) and noxious mechanical (15–60 g, pinch) stimulation following diclofenac administration in inflamed rats are shown in Figure 3(d). After daily administration of diclofenac over 2 days in inflamed rats, the discharge frequency of SpVc WDR neurons to both non-noxious and noxious mechanical stimulation decreased to control levels (Figure 3(d)). The lowered mechanical threshold, and augmented spontaneous and noxious and non-noxious firing frequencies in inflamed rats returned to that observed in naïve rats.

As shown in Figure 4(a), the mean discharge frequency of SpVc WDR neurons in inflamed rats significantly decreased after diclofenac administration for both non-noxious and noxious mechanical stimuli ($p < 0.05$). The mean mechanical stimulation threshold in inflamed rats after diclofenac also significantly reversed to control levels (Figure 4(b)). Spontaneous discharge of SpVc WDR neurons in inflamed rats also significantly decreased after diclofenac administration (Figure 4(c), $p < 0.05$). The mean receptive field size in inflamed rats was significantly decreased to control levels (Figure 4(d)).

Discussion

Administration of quercetin attenuates trigeminal inflammatory hyperalgesia

Previous studies have indicated that quercetin administration attenuates nociceptive behavior in the neuropathic pain model.[30,31] CFA-inflamed models are generally well-established for trigeminal chronic pain investigations.[1,5,6] In the present behavioral study, we found the following: (i) a significantly lower threshold of escape from mechanical stimulation applied orofacially in CFA-inflamed rats compared to naïve rats, as reported previously[10]; (ii) a reversal of the reduced mechanical threshold to control levels in inflamed rats on day 2 of chronic quercetin administration; and (iii) vehicle administration had no significant effect on the escape threshold in day 2 inflamed rats. A previous study indicated intraperitoneal administration of 50 mg/kg quercetin inhibits Cox-2 expression in the renal inner medulla in response to ureteral obstruction and is associated with inflammation in the renal parenchyma.[21] As such, in this study we examined whether systemic application of 50 mg/kg quercetin could attenuate inflammatory hyperalgesia. Application of this dose of quercetin significantly attenuated hyperalgesia after only 2 days of inflammation. This agrees with previous findings of a significantly decreased CFA inflammation-induced mechanical hyperalgesia in a rat inflammatory pain model, after application of a dietary constituent, such as polyphenol and carotinoid.[10–13] Although the precise mechanism underlying the effects of quercetin on inflammation-induced hyperalgesia remains unknown, several possibilities exist. Quercetin decreases the production of PGE_2 by inhibiting Cox-2 cascades.[20–22] Together, these observations suggest daily quercetin use reduces inflammation-induced hyperalgesia in whisker pads via Cox-2 suppression resulting in inhibition of PGE_2 production possibly via previously described mechanisms[12]

Quercetin suppresses the hyperexcitability of SpVc WDR neuronal activity associated with hyper algesia following inflammation

The generally accepted mechanism of nociceptive sensory signaling depends on the following four general processes: first, transduction from peripheral terminals that transduce external stimuli; second, generation of action potentials; third, propagation of action potentials along axons; and fourth, transmission to central terminals, which form the presynaptic elements of the first synapses of the central nervous system sensory pathways[1,32] In this study, systemic administration of quercetin reversed the decreased mean mechanical stimulation threshold in inflamed rats, with both the non-noxious and noxious mechanical stimuli-evoked mean discharge frequency of SpVc WDR neurons returning to control levels in inflamed rats after quercetin treatment. The proinflammatory mediator, PGE_2 binds to G protein-coupled prostanoid EP receptors and can activate protein kinase A in nociceptive peripheral terminals following peripheral inflammation.[33] Protein kinase A then leads to phosphorylation of mechanosensitive transient receptor potential ankyrin 1 channels and Nav and Kv channels. As a result, the activation threshold for transient receptor potential ankyrin 1 transducer channels is decreased and membrane excitability increases in peripheral terminals. These events result in a higher frequency of nerve impulses being conducted to presynaptic central terminals of the SpVc. Therefore, our findings indicate that systemic quercetin may modulate inflammation-induced peripheral sensitization and SpVc WDR neuronal hypersensitivity in peripheral nerve terminals, as suggested by previous in vitro findings in neuronal activities via modulation of Nav and Kv channels.[18,19] Moreover, the present study showed that quercetin reversed the increased mean spontaneous discharge frequency of SpVc WDR neurons following inflammation.

Burstein et al.[34] reported that the ongoing activities observed in the SpVc are responsible for ongoing headache (spontaneous pain). The origin of ongoing activity in the central neurons that relay sensory information is of considerable clinical interest because it has been suggested as a determinant of the level of post-traumatic injury and chronic pain.[35] A more recent study demonstrated that ongoing activity of WDR neurons in the SpVc is driven from the periphery, because microinjection of lidocaine into the trigeminal ganglia

causes a significant decrease in ongoing activity.[36] Together with the present results, this suggests quercetin attenuates the increased spontaneous discharge activity of SpVc WDR neurons innervating the whisker pad resulting from peripheral and/or trigeminal ganglion sensitization.

We previously reported that a local GABAergic mechanism could control nociceptive transmission in SpVc neurons, thus impacting the overall properties of mechanical receptive fields.[29] In the present study, the expanded receptive field size in inflamed rats returned to control levels after quercetin administration, although the mechanisms underlying this effect remain unclear. A previous study showed that quercetin produces dose-dependent antinociception in several models of chemical pain via mechanisms involving the GABAergic system.[37] Thus, it is possible that quercetin modulates local GABAergic tonic control of nociceptive mechanoreceptive transmission and inhibits central mechanisms through excitatory synaptic transmission. In the present study, the mean receptive field size in the inflamed rats was relatively larger than that of previous study.[10] Previously, we reported that the changes in mechanical receptive field size was local iontophoretic application of $GABA_A$ receptor agonist and antagonist.[29] Although the mechanisms underlying this difference remain unclear, it can be assumed that mean receptive field size of inflamed SpVc neurons under pentobarbital anesthesia (potentiates the GABAergic inhibition) was relatively smaller than that of under the combination of three anesthetic agents. However, further studies needed to confirm this possibility.

Lack of evidence for noxious pinch-evoked afterdischarges of SpVc neurons following inflammation

Previous studies reported "noxious pinch-evoked afterdischarge" in SpVc WDR neurons undergoing noxious mechanical stimulation in a chronic inflammation neuropathic model under pentobarbital anesthesia. The study associated these changes with neuronal sensitization during persistent pain,[10–13,38] suggesting noxious pinch-evoked afterdischarge is an important phenomenon of hyperexcitability of noxious neurons in pathological pain. In this study however, no obvious noxious pinch-evoked afterdischarges were observed in CFA-inflamed rats under a mixture of three anesthetic agents, compare with the previous pentobarbital-anesthetized rats. While the precise mechanism remains unclear, the most probable explanation for this difference is likely due to the different anesthetic agents used. Although it is generally known that pentobarbital anesthesia modulates $GABA_A$ receptor-mediated chloride channel gating, pentobarbital anesthesia potentiates the inhibitory GABAergic interneuron in the SpVc.[39] The mechanisms underlying the combination mixture of three anesthetic agents are as follows; i) medetomidine is a potent and selective α_2-adrenoceptor agonist; ii) midazolam is a benzodiazepine that enhances the inhibitory action of GABAergic interneurons; iii) butorphanol exhibits agonist activity at the μ-and κ-opioid opioid receptors in opioidergic inhibitory interneurons.[40,41]

In relation to the mechanism underlying "noxious pinch-evoked afterdischarge" in inflamed conditions, Radhakrishnan et al.[42] showed that administration of a substance P (SP) neurokinin-1 (NK_1) receptor antagonist inhibits pinch-evoked afterdischarges in WDR neurons of the spinal cord. Therefore, from the results of the current study, the following two speculations could be made. Firstly, that noxious pinch-stimulation of an inflamed sensitized receptive field generates a large number of action potentials in peripheral terminals, that then propagate to the central terminal of trigeminal ganglion neurons, open CaV channels and release large amounts of glutamate in the synaptic cleft, which binds to postsynaptic glutamate receptors and thereby augments the discharge frequency of SpVc WDR neurons in inflamed rats. Secondly, the large number of action potentials also propagates to other branches of the central terminal of trigeminal ganglion neurons, activating excitatory SP-containing interneurons that then release SP into the synaptic cleft, which binds to postsynaptic NK1 receptors in SpVc WDR neurons in inflamed rats and triggers noxious pinch-evoked afterdischarges.

In a previous study, we demonstrated that using multi-barrel electrodes, iontophoretic application of glutamate evoked cervical dorsal horn neuronal activity responding to noxious stimulation of the trigeminal area. This glutamate-evoked neuronal activity was also current-dependently inhibited by iontophoretic application of the α_2-adrenoceptor agonist, clonidine.[43] Since then, single-cell reverse-transcription polymerase chain reaction analysis has shown smalldiameter noxious trigeminal ganglion neurons express α_2-adrenoceptor mRNA.[44] In the current study, one of the anesthetic agents used in the mixture is medetomidine, a selective α_2-adrenoreceptor agonist that presynaptically inhibits SP release from the central nerve terminal of excitatory interneurons *via* the α_2-adrenoreceptor. Therefore, its effect may be confounding the effects on noxious pinch-evoked afterdischarges in inflamed SpVc WDR neurons under the three anesthetic agents compared with pentobarbital anesthesia in inflamed rats. However, further studies are needed to confirm this possibility.

Functional significance of the suppressive effect of quercetin on the hyperexcitability of SpVc neurons associated with hyperalgesia

It is well known that acidic antipyretic analgesic NSAIDs are potent and efficient inhibitors of Cox-2 for analgesic drugs.[45] Current options for the pharmacological treatment of pain include NSAIDs and opioids, which unfortunately causes several side effects including an increased risk of stomach ulcers and heart attacks.[45] NSAIDs are of increased interest as CAMs for the treatment of persistent chronic pain.[23,24] We

have previously examined whether systemic administration of dietary constituents, including polyphenols, polyunsaturated fatty acids, and carotenoids, attenuates inflammation-induced mechanical hyperalgesia, primarily by suppressing SpVc WDR neuronal hyperexcitability via both peripheral and central Cox-2 cascade signaling pathways.[10-13] However, we did not compare the inhibitory effect of hyperalgesia associated with hyperexcitability of nociceptive neurons between dietary constituents and NSAIDs.

The present behavioral study showed the following: (i) reversal of the reduced mechanical threshold to control levels in inflamed rats on day 2 of chronic diclofenac administration; (ii) the mean discharge frequency of SpVc WDR neurons to both non-noxious and noxious mechanical stimuli in inflamed rats significantly decreased after diclofenac administration; (iii) the increased mean spontaneous discharge of SpVc WDR in inflamed rats significantly decreased after diclofenac administration; and (iv) diclofenac restored the expanded mean receptive field size in inflamed rats to control levels. In this study, the magnitude of quercetin-mediated inhibition on the hyperexcitability of SpVc neurons associated with hyperalgesia was almost equal to that of diclofenac (50 mg/kg, i.p.), suggesting quercetin is comparable to diclofenac, and is a potential therapeutic agent for CAM strategies for preventing trigeminal inflammatory mechanical hyperalgesia.

It is clinically generally known that most patients undergoing orthodontic treatment complain of pain, including referred pain, and NSAIDs are often administered for the relief of pain symptoms. Tooth movement is achieved through mechanical forces provided by orthodontic appliances. During orthodontic treatment, periodontal inflammation processes and bone absorption on the tension side are observed.[46] Since PGE_2 has an important contribution to the function of osteoclast-related bone remodeling, NSAIDs produce negative effects in orthodontic patients, such as reduction of tooth displacement.[47,48] Recently, we have shown that chronic administration of the polyphenol, resveratrol, attenuates experimental tooth movement-induced, mechanical, ectopic hyperalgesia that is associated with hyperexcitability of SpVc WDR neurons in anesthetized rats. These results suggest that this dietary constituent is a potential therapeutic analgesic agent for ectopic pain, such as the pain of orthodontic patients.[49] These findings suggest that administration of quercetin may also attenuate orthodontic treatment-induced ectopic hyperalgesia without any side effects.

Together, this is first study that compares the suppressive potency of quercetin and the NSAID diclofenac in inflammation-induced SpVc neuronal excitability associated with hyperalgesia. Therefore, these results contribute to the development of analgesic drugs for the treatment and prevention of trigeminal inflammatory pathological pain, including clinical orofacial pain, with fewer side effects.

Conclusion

These results suggest that quercetin administration attenuates inflammatory hyperalgesia associated with hyperexcitability of nociceptive SpVc WDR neurons via inhibition of the peripheral Cox-2 signaling cascade and voltage-gated ion channels. These findings support the proposed potential of quercetin as a therapeutic agent in CAM strategies for preventing trigeminal inflammatory mechanical hyperalgesia.

Author contributions

HI performed all experiments, analyzed data, and prepared figures. RY helped with behavioral and electrophysiological experiments. MT participated in the design of the experiments and wrote the manuscript. All the authors have read and approved the paper.

Declaration of Conflicting Interests

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