TNFα Mechanically Sensitizes Masseter Muscle Afferent Fibers of Male Rats

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Abstract

Behavioral evidence in rats indicates that injection of tumor necrosis factor alpha (TNFα) into skeletal muscle results in a prolonged mechanical sensitization without gross inflammation. To investigate whether a peripheral mechanism could underlie this effect, in the present study, TNFα (1 or 0.1 μg) was injected into the rat masseter muscle to assess its effect on the excitability and mechanical threshold (MT) of muscle nociceptors as well as on inflammation. Expression of TNFR1 (P55 receptors) and TNFR2 (P75 receptors) by the masseter muscle and trigeminal ganglion neurons that innervate that muscle was determined by Western blot and immunohistochemistry, respectively. The Evans blue dye technique was used at the end of the TNFα experiments to assess for plasma protein extravasation. In subsequent experiments to confirm the involvement of receptor activation in TNFα-induced effects, P55 receptor or P75 antibody was co-injected with TNFα. Intramuscular injection of 1 μg TNFα did not excite nociceptors, but did significantly decrease MT compared to vehicle control. There was no evidence of gross inflammation 3 hours after injection of TNFα. Co-injection of TNFα with P55 or P75 receptor antibodies attenuated TNFα-induced mechanical sensitization. P55 and P75 receptors were expressed by 29% and 62% of masseter nociceptors, respectively. These findings indicate that TNFα induces mechanical sensitization of masseter nociceptors that is mediated through activation of peripheral P55 and P75 receptors. These results support the hypothesis that a peripheral receptor mechanism could contribute to TNFα-induced non-inflammatory mechanical sensitization of skeletal muscle previously reported in behaving rats.
**Introduction**

TNFα is a proinflammatory cytokine released from different types of cells such as macrophages, monocytes, lymphocytes, neutrophils and fibroblasts (Baud and Karin 2001). Schwann cells in the peripheral nervous system also release TNFα upon injury (Wagner and Myers 1996). Although TNFα produces its biological effects by binding two receptors, the TNFR1 or P55 receptor, and the TNFR2 or P75 receptor (Vandenabeele et al. 1995), previous animal studies have suggested that P55 receptor activation was responsible for TNFα-induced cutaneous hyperalgesia (Cunha et al. 2005; Jin and Gereau 2006; Parada et al. 2003; Sommer et al. 1998). TNFα plays an important role in inflammatory processes and for example, synovial fluid TNFα levels are elevated in patients suffering from various arthritic diseases such as rheumatoid arthritis and ankylosing spondylitis (Chu et al. 1991; Feldmann et al. 1996; Francois et al. 2006; Lange et al. 2000; Nordahl et al. 2000). Use of anti-TNFα treatment reduces joint pain in these conditions and is considered one of the major breakthroughs in the management of pain and inflammation (Boettger et al. 2008; Kopp et al. 2005; Lipsky et al. 2000; Moen et al. 2005).

In contrast, a number of chronic muscle pain conditions, such as fibromyalgia and myofascial temporomandibular disorders (TMD), are not associated with clinical signs and symptoms of tissue inflammation and thus it is unclear what role, if any, TNFα might play in these conditions. However, serum TNFα levels are elevated in patients suffering from fibromyalgia (Wang et al. 2008). Previous animal studies in the rat have shown that subcutaneous and intraplantar injection of TNFα caused sensitization and inflammation (Cunha et al. 1992; Junger and Sorkin 2000; Woolf et al. 1997). Moreover, subcutaneous injection of TNFα evoked activity in 14% of putative nociceptive C-fibers, but did not evoke afferent fiber discharge in
mechanosensitive Aβ fibers (Junger and Sorkin 2000), which suggests that TNFα may be
selective for slowly-conducting cutaneous afferent fibers with nociceptive properties. In a
subsequent behavioural study, intramuscular injection of TNFα into the gastrocnemius muscle
was also reported to induce mechanical sensitization without gross inflammation, although it did
increase levels of pro-inflammatory mediators, such as prostaglandin E2 (PGE2), calcitonin gene
related peptide (CGRP) and nerve growth factor (NGF) (Schafers et al. 2003). Despite the
elevation of these substances after intramuscular injection of TNFα, a previous study reported
that TNFα had little effect on the excitability or mechanical sensitivity of C-fibers that innervate
the gastrocnemius muscle of the rat (Hoheisel et al. 2005). The reason for these apparent
contradictory findings is uncertain, however, these results suggest that the role TNFα plays in
non-inflammatory myofascial pain mechanisms remains to be determined.

The purpose of this study was to elucidate the peripheral effect(s) of TNFα on a different
muscle often associated with chronic muscle pain conditions, the masseter (jaw closer) muscle.
To do this, the activity of individual masseter muscle afferent fibers was recorded in vivo in the
rat and the effect of intramuscular injection of TNFα on the excitability and MT of these afferent
fibers was monitored. It was hypothesized that intramuscular injection of TNFα into rat masseter
muscle would cause a decrease in the MT of masseter muscle afferent fibers without gross
inflammation and that this effect would be mediated, at least in part, through activation of P55
receptors. To determine whether TNFα could exert direct effects on masseter muscle afferent
fibers, additional immunohistochemical and Western blot experiments were undertaken to
determine the extent of TNFα receptor expression by masseter muscle afferent fibers and in the
masseter muscle, respectively.
Materials and Methods

Animals

A total of 77 male adult Sprague-Dawley rats (260-380g) were used in this study. All experiments were done in accordance with the Canadian Council on Animal Care and were approved by the University of British Columbia Animal Care Committee.

Immunohistochemistry

Immunohistochemistry was performed in 5 male rats to determine the expression of P75 and P55 receptors on trigeminal ganglion neurons that innervate the masseter muscle. Fast blue (2%, Polyscience, USA), which is a fluorescent dye, was injected bilaterally into the masseter muscles of five male rats to identify masseter ganglion neurons. After seven days, rats were euthanized and perfused with heparinised saline followed by 4% paraformaldehyde. The right and left trigeminal ganglia were removed and each ganglion cut into 6-8 40 μm thick sections with a vibratome and processed for indirect immunofluoresence immunohistochemistry as described earlier (Dong et al. 2007; Sung et al. 2008). Briefly, sections were incubated with 5% normal goat serum (NGS) in phosphate buffered saline (PBS) for 1 hour and then incubated overnight with anti-rat P55 receptor antibody (1:1000; Rabbit polyclonal, Abcam, USA) or anti-rat P75 receptor antibody (1:20000; Rabbit polyclonal, Sigma-Aldrich, USA). The next morning, sections were extensively washed with phosphate-buffered saline (PBS) and then incubated with CY3 conjugated IgG (Anti-rabbit; Jackson Immunoresearch,USA) for one hour at room temperature in the dark. After several washings with PBS, sections were mounted on slides with
cover slips and visualized with a Leica DM L fluorescent microscope. All sections were examined and all fast blue positive cells counted. WCIF Image J software program (NIH Image, USA) was used to calculate the area of each fast blue positive cell including nucleus. In control experiments, the tissue sections were incubated without primary antibody or with a combination of primary antibody and P55 receptor peptide (Abcam, USA; preabsorption) to confirm specificity of receptor-like immunoreactivity, respectively.

Electrophysiology

Adult male Sprague Dawley rats (260-380g, n=70) were used for acute in vivo recording of masseter muscle afferent fiber activity as has been previously reported in detail (Cairns et al. 2002; Dong et al. 2007; Mann et al. 2006). Rats were anesthetized with isoflurane (2-2.5%). The carotid artery was cannulated to monitor blood pressure and to administer Evans blue dye and pentobarbital (100 mg/kg) at the end of the experiment to test for gross inflammation and to sacrifice the rat, respectively. Rat core body temperature, expired CO2, heart rate and blood pressure were monitored throughout the experiment and were kept within the range of 36.8-37.1 °C, 20-50 mm Hg, 300-400 beats/min and 60-80 mm Hg, respectively. Depth of isoflurane anesthesia was assessed by periodically checking for the absence of a leg withdrawal reflex in response to strong pressure applied to the toes with a pair of small forceps. If this nociceptive reflex response was present, or if the mean blood pressure and or heart rate exceeded 90mm Hg or 430/min, respectively, the concentration of inhaled isoflurane was increased until the reflex disappeared and the other parameters return to their desired ranges (see above). In this manner, adequate depth of anesthesia was maintained.

A parylene-coated tungsten-recording electrode was introduced into the trigeminal ganglion to record from individual masseter afferent fibers. Masseter afferent fibers were
identified by their response to blunt probing of the masseter muscle (Cairns et al. 2002; Cairns et al. 2001). When an afferent fiber was found that appeared to respond to mechanical stimulation of the muscle, the skin overlying the muscle was pulled and pinch, and pressure stimuli were applied to the skin to check that the mechanoreceptive field of the afferent fiber was not in the skin (Cairns et al. 2002; Cairns et al. 2001). The antidromic collision technique was used to confirm the projection of masseter muscle afferent fibers to the caudal brain stem (Cairns et al. 2002; Cairns et al. 2001). To produce antidromic action potentials, constant current electrical stimuli were applied to a stimulating electrode lowered into the caudal brain stem. Orthodromic action potentials were evoked by mechanically stimulating the masseter muscle fibers with a probe. A projection to the caudal brainstem was confirmed by a collision between the orthodromic and antidromic action potentials. At the end of the experiment, the estimated conduction velocity of each fiber was calculated by dividing the distance between stimulating and recording electrodes by the latency of the antidromic action potential. As a final confirmation that the afferent fiber was indeed in the muscle, hypertonic saline (HS; 1M) was injected into the mechanoreceptive field of each fiber at the end of experiment.

**TNFα induced mechanical sensitization**

Experiments were carried out to determine the effect of intramuscular injection of two different doses (0.1μg,1μg) of TNFα on masseter muscle afferent fiber excitability and mechanical sensitivity. Before the injection of any substance, baseline afferent fiber MT was measured with an electronic von Frey Hair (model 160IC, IITC, California, USA) at one-minute intervals for 10 minutes (Cairns et al. 2007; Cairns et al. 2002; Mann et al. 2006). The baseline MT was determined by averaging ten consecutive mechanical stimuli. Each afferent fiber
identified was randomly assigned to injection of one of the following groups: vehicle control (10 μL PBS, n = 10), 1 μg TNFα (Sigma, USA; 1 μg in 10 μl PBS, n = 10) and 0.1 μg TNFα (Sigma, USA; 0.1 μg in 10 μl PBS, n = 10). The investigator (AH) was blinded to the content of injections. The dose of TNFα was the same as in a previous rat behavioral study (Schafers et al. 2003). Solutions were injected intramuscularly with a 26 gauge needle connected by polyethylene tubing to a 25 μl Hamilton syringe. The needle was inserted into the mechanoreceptive field of the masseter muscle afferent fiber, then baseline afferent fiber activity was recorded for 10 minutes prior to the first injection to record any spontaneous fiber activity before the injection. At the end of the 10-minute baseline recording, 10 μl of solution was injected into masseter muscle and evoked activity was monitored for 10 minutes. MT was recorded every hour for 10 minutes for a total of 3 hours after substance injection. At the end of MT recording, HS was injected into the mechanoreceptive field of the masseter afferent fiber as positive control. Afferent fiber discharge in response to injection of HS confirmed that the afferent fiber being recorded innervated the masseter muscle and could respond to a noxious stimulus, thus identifying it as a putative masseter nociceptor. Finally, to assess whether injection of TNFα resulted in gross inflammation of the masseter muscle, Evans blue dye (6 mg/kg) was injected via the carotid artery (Mann et al. 2006). Approximately 15 minutes after Evans blue dye injection, the rat was perfused and the part of the masseter muscle around the site of injection was removed for Evans blue dye analysis.

Formalin Injection

Formalin (37%, 10 μl) was injected into the masseter muscle as positive control. Three hours after injection, the Evans blue dye method as described in the previous section was used to assess plasma protein extravasation.
Evans blue dye analysis

After removal, muscle tissues were weighed and placed in test tubes containing 2 ml of formamide. Test tubes were incubated at 60 °C for 24 hours. After 24 hours, the supernatant was collected and the amount of dye extracted from the muscle was determined by measuring the absorbance of supernatant at 620 nm using a spectrophotometer (Fiorentino et al. 1999). The concentration of dye was calculated per gram weight of muscle tissue.

Receptor pharmacology

Additional experiments were carried out to determine the effect of P55 and P75 receptor antagonism on the effect of TNFα. In these experiments, 1μg of P55 receptor antibody (Abcam, USA) was injected alone (control) or co-injected with 1μg TNFα into the mechanoreceptive field of the masseter afferent fiber. Similarly, 1.2 μg of P75 receptor antibody (Sigma-Aldrich, USA) was injected alone or co-injected with 1μg TNFα. The investigator (AH) was blinded to the content of injections. Baseline and post injection MTs were recorded as described above in the TNFα experiments. The dose of antibodies was determined in vitro by using the Western blot technique to determine antibody-binding saturation in the masseter muscle.

Western blot analysis

Western blot experiments were carried out as previously described (Billova et al. 2007). Briefly, rat masseter muscles were taken and weighed. Muscles were homogenized on ice using lysis buffer. Homogenised muscle was centrifuged at 14000 rpm for 30 min at 4 °C, the supernatant was collected and its total protein content was determined using the Bradford method.
Protein samples (20 μg) were separated by electrophoresis using 7% SDS polyacrylamide gel and were transferred to a 0.2 μm nitrocellulose membrane. The membrane was blocked with 5% non-fat dried milk and was cut and treated with different concentrations of P55 receptor antibody or P75 receptor antibody at 4 °C overnight. The next morning membranes were washed and treated with the secondary antibody (Goat anti-rabbit peroxide conjugated; Jackson Immunoresearch, USA) for 1 hour at room temperature. After washing, the bands were detected with ECL Western blotting detection chemiluminescence reagent (Amersham™, GE Health Care, UK) and analysed by Alpha Innotech Imager FluorChem 8800 (Alpha Innotech Co; San Leandro, CA) gel box imager. Band density was measured by using FluorChem software (Alpha Innotech Co.).

Data analysis

Immunohistochemistry

Fast blue positive masseter ganglion neurons were examined under the Leica DML fluorescent microscope. P55 and P75 receptor-like immunoreactivity was examined in fast blue positive cells. WCIF Image J software program (NIH Image, USA) was used to measure the area of fast blue positive cells. The percentage of P55 and P75 labelled masseter ganglion neurons was calculated.

Electrophysiological experiments

The average of ten afferent fiber MTs at each time point was calculated. Relative MTs were then calculated by dividing mean MT at each time point by the mean baseline threshold. Cumulative discharge was defined as the difference between the number of action potentials
recorded before and after injection of substances into the masseter muscle. Cumulative discharge was calculated by subtracting the number of action potentials recorded for 10 minutes before injection (baseline) from the number recorded for 10 minutes after injection.

**Statistics**

Significant differences in the mean expression of P75 and P55 receptors by masseter ganglion neurons in the 5 male rats were assessed with a paired t-test. The distribution of expression frequencies amongst the various sizes of masseter ganglion neurons for P75 and P55 receptors was compared with a Chi-square test.

A sample size estimate employing analysis of variance (ANOVA) suggested that samples of 10 afferent fibers per group would permit detection of a 50% difference in afferent fiber MT between the TNFα and control groups with α equal to 0.05 and a power of 0.80. A 2-way, repeated measures ANOVA test was used to determine the effect of all treatments (vehicle, TNFα (1μg, 0.1μg), TNFα (1μg) coinjected with P55 receptor antibody, TNFα (1μg) coinjected with P75 receptor antibody or P55 or P75 receptor antibody alone) and time (repeated) on relative MTs. A logarithmic transformation of the MT data was performed to produce equal variance prior to analysis with ANOVA. Post-hoc comparisons were undertaken with the Holm-Sidak test. Since the afferent fiber discharge data was not normally distributed, Kruskal Wallis ANOVA on ranks was employed to determine whether there were significant differences in the median afferent fiber discharge evoked by vehicle control, TNFα (1μg, 0.1μg), TNFα (1μg) coinjected with either P55 or P75 receptor antibody, or P55 or P75 receptor antibody alone (control). Correlation analyses were undertaken with Pearson product moment test. Alpha <0.05 was considered significant.
Results

Immunohistochemistry

Masseter muscle ganglion neurons were examined for expression of P55 (n=688 cells) and P75 (n =503 cells) receptor-like immunoreactivity in 5 male rats (Figure 1A). The mean (± standard error of the mean) percentage expression of P75 receptors was significantly higher (62 ± 1%) than P55 receptors (29 ± 2%) in masseter ganglion neurons. Receptor-like immunoreactivity was uniformly distributed amongst small, medium and large masseter muscle ganglion neurons without any discernable differences in fluorescence intensity in both P75 and P55 receptors (Figure 1B). There was no labeling in the preabsorption studies or in the absence of the primary antibody.

Afferent fiber properties

Single unit recordings from 70 masseter muscle afferent fibers (n=70 rats) that projected to the caudal brainstem were made (Figure 2A). The conduction velocity (CV) of 66 of these afferent fibers was in the Aδ (CV = 2-12 m/s) range, 1 in the Aβ (CV > 12 m/s) range and 3 in the C-fiber (CV < 2 m/s) range. The percentage of C-fiber mechanoreceptors recorded (4%), though relatively low, is consistent with previous studies (Cairns et al. 2003). An example of MT determination in an individual masseter afferent fiber is shown in Figure 2B. The mean baseline MT of these fibers was 35± 3 g. HS was used as positive control to confirm that the mechanoreceptive field of each afferent fiber was in masseter muscle (Figure 2C). HS evoked afferent fiber discharge from all the fibers included in the study. The combination of slow conduction velocity, relatively high MT and response to a noxious chemical stimulus indicate that all recorded afferent fibers could be classified as putative muscle nociceptors (Cairns 2008.)
Effect of TNFα on evoked activity and MT

The median [interquartile range] cumulative discharge evoked by TNFα (1μg: 0[-2.2-1.5] spikes, 0.1μg: 0-[0-0.75] spikes) was not significantly different from control (0 [0-3.5] spikes).

Mean baseline MT was 24.2 ±5.2 g, 26.3±4.8 g, and 19.1±6.4 g, respectively for the 1μg TNFα, 0.1 μg TNFα and control group. Two-way repeated measures ANOVA on all the MT data indicated a significant effect of treatment and time as well as a significant interaction between time and treatment. Post-hoc evaluation revealed that there was a significantly greater effect of treatment with 1μg TNFα than vehicle on MT, and that a significant difference between the treatment and control group occurred at 180 minutes post-injection (Figure 3). These results indicate that there was a time-related mechanical sensitization of masseter nociceptors that occurred independent of treatment, but that treatment with TNFα produced a significantly greater mechanical sensitization than vehicle or 0.1μg TNFα at 180 minutes post-injection.

Evans blue dye analysis

Evans blue dye was used to assess the extent of plasma protein extravasation into the masseter muscle 3 hours after TNFα (0.1 or 1μg) or vehicle control injections. There was no significant difference between the amount of Evans blue dye in the masseter muscle when the vehicle control and TNFα (0.1 or 1μg) group were compared (Figure 4). In contrast, the amount of dye absorbed in the masseter muscle 3 hours after 37% formalin injection was 22±1μg/g. These results indicate that TNFα did not cause greater inflammation than vehicle control when injected into the masseter muscle.
Determination of P55 and P75 receptor antibody concentration

To determine an appropriate concentration of P75 and P55 receptor antibodies with which to test the involvement of these receptors in TNFα-induced mechanical sensitization, Western blot experiments were undertaken to assess the concentration at which the antibody binding was saturated in masseter muscle homogenate. Bands were detected at 55 kDa and 75 kDa, which correspond to P55 and P75 receptors, respectively (Figure 5A). It was found that at concentrations 1μg/ml (Houzen et al. 1997) and 1.2μg/ml, the binding of the P55 receptor (Figure 5B) and P75 receptor antibodies, respectively, to the masseter muscle homogenate were effectively saturated. Western blot experiments also confirmed the expression of both receptors in masseter muscle tissue.

Effect of P55 and P75 receptor antibodies on evoked activity and TNFα-induced mechanical sensitization

TNFα receptor antibodies were used to confirm the involvement of P55 or P75 receptors in TNFα-induced mechanical sensitization of masseter muscle nociceptors. Co-injection of P55 receptor antibody and 1μg TNFα into the masseter muscle evoked median (interquartile range) cumulative nociceptor discharge (0 [0-2.25] spikes, n=10) that was not significantly different from that evoked by P55 receptor antibody alone (control; 1[0-16.5] spikes, n=10). Similarly, co-injection of P75 receptor antibody and 1μg TNFα into the masseter muscle evoked cumulative nociceptor discharge (0[0-104.5] spikes, n=10) that was not significantly different from that evoked by P75 receptor antibody alone (control; 0[0-4.5] spikes, n=10). Mechanical sensitization induced by 1μg TNFα with P55 receptor antibody was not significantly different from vehicle control (Figure 6A). However, when 1μg TNFα with P75 receptor antibody was
injected, the relative MT was significantly increased compared to the vehicle control and 1μg
TNFα alone (Figure 6B). These results indicate that both P55 and P75 receptors play a role in
TNFα–induced mechanical sensitization of masseter muscle nociceptors.

Discussion

Injection of TNFα into the masseter muscle did not excite nociceptors but 1μg TNFα did
induce a delayed mechanical sensitization that was mediated through activation of both P55 and
P75 receptors. Subsequent injection of the algogen HS into the masseter muscle evoked discharge
in all nociceptors. Thus, TNFα appears to induce mechanical sensitization in masseter muscle
nociceptors. Immunohistochemical and Western blot results confirmed that P55 and P75
receptors are expressed by masseter ganglion neurons and in the masseter muscle, respectively.
Together, these results suggest that TNFα-induced changes in muscle sensitivity previously
reported in behaving rats (Schafers et al. 2003) occur, in part, due to mechanical sensitization of
muscle nociceptors through activation of peripheral P55 and P75 receptors.

Injection of 1μg TNFα into the masseter muscle induced a slowly developing mechanical
sensitization of nociceptors that became significant 3 hours post-TNFα injection. This finding is
consistent with previous animal studies that have demonstrated that subcutaneous injection of
TNFα produced behavioural evidence of mechanical and thermal hyperalgesia in the rat paw
within a few hours (Cunha et al. 1992; Jin and Gereau 2006; Woolf et al. 1997). Subcutaneous
injection of 5ng TNFα has been shown to result in the mechanical sensitization of C-fibers as
well as to induce cutaneous inflammation, which suggests that part of the sensitizing effect of
TNFα in the skin could be due to the release of other inflammatory mediators (Junger and Sorkin
2000). Behavioural studies in rats also indicated that intramuscular injection of 10μg TNFα could
induce a prolonged mechanical sensitization of the gastrocnemius muscle (Schafers et al. 2003). However, intramuscular injection of 0.25μg TNFα was not demonstrated to increase the response of gastrocnemius muscle C-fibers to mechanical stimulation (Hoheisel et al. 2005). This apparent discrepancy between the results obtained in behavioural and afferent fiber recording studies is likely due to the substantially smaller dose of TNFα employed in the C-fiber recording experiments and to the fact that afferent fiber mechanical response was only assessed for 10 minutes after the injection of TNFα into the gastrocnemius muscle (Hoheisel et al. 2005). In the current study, it was found that several hours were required for significant mechanical sensitization of masseter nociceptors to become apparent.

The findings of the present study demonstrate the expression of both P55 and P75 receptors on masseter muscle ganglion neurons and within the masseter muscle. Previous studies have shown the expression of both P55 and P75 receptors in the trigeminal ganglion (Cunningham et al. 1997), skeletal muscle (Li and Reid 2001), on Schwann cells (Qin et al. 2008), dorsal root ganglion neurons (Hensellek et al. 2007,; Shubayev and Myers 2001) and the P55 receptor in the trigeminal mesencephalic nucleus (Bette et al. 2003). Taken together, these findings suggest that TNFα could potentially induce some of its effects through a direct action on masseter nociceptors. Animal studies have shown that P55 receptors contribute to TNFα-induced cutaneous hyperalgesia (Cunha et al. 2005; Jin and Gereau 2006; Parada et al. 2003; Sommer et al. 1998). However, recent studies have shown the involvement of P75 receptors in tumor-induced heat hyperalgesia by TNFα in dorsal root ganglion neurons (Constantin et al. 2008) and in the maintenance of neuropathic pain (Schafers et al. 2008). While activation of either P55 or P75 receptors appears sufficient to induce mechanical sensitization of masseter muscle nociceptors, the expression of P55 receptors was only observed in about one third of masseter
ganglion neurons. In contrast, the expression of P75 receptors was much higher (62%). This higher expression level of P75 receptors by masseter muscle ganglion neurons suggests that any direct action of TNFα on masseter muscle nociceptors to induced mechanical sensitization was more likely due to activation of P75 receptors.

The low level of P55 receptor expression by masseter ganglion neurons and presence of both receptors in masseter muscle tissue suggests that TNFα could also operate indirectly through activation of non-neuronal P55 and/or P75 receptors to induce the release of other sensitizing mediators (Schafers et al. 2003; Woolf et al. 1997). This concept is supported by the finding that in the current as well as a previous study (Schafers et al. 2003), TNFα-induced mechanical sensitization of skeletal muscle required several hours to develop. The expression of TNFα receptors by non-neuronal cells such as muscle cells (Li and Reid 2001) and previous findings that TNFα induced the release of various sensitizing substances such as PGE2, NGF and CGRP (Schafers et al. 2003; Woolf et al. 1997) further support the hypothesis that TNFα could act indirectly to mechanically sensitize masseter nociceptors. For example, recent evidence indicates that TNFα indirectly suppresses potassium currents to increase the excitability of rat dorsal root ganglion neurons through an increase in PGE2 (Liu et al. 2008). Intramuscular injection PGE2 has been shown to enhance the response of cat gastrocnemius muscle C-fibers to the action of bradykinin (Mense 1981) and to mechanically sensitize rat cutaneous nociceptors (Chen et al. 1999) and masseter muscle nociceptors (unpublished data from our laboratory). PGE2-induced sensitization is mediated in part through EP2 and EP3 receptors that have recently been shown to be expressed by trigeminal nociceptors (Patwardhan et al. 2008), which suggests that a TNFα-induced release of PGE2 could have sensitize masseter nociceptors to mechanical stimulation in the present study. Intradermal and intramuscular injection of TNFα also elevates
tissue concentrations of NGF, however, in one study injection of rat NGF into rat masseter muscle did not appear to induce significant afferent fiber mechanical sensitization (Mann et al. 2006). On the other hand, injection of human NGF has been shown to mediate a prolonged mechanical sensitization of the human masseter muscle and appears to also mechanically sensitize rat masseter muscle nociceptors (Svensson et al. 2003; Svensson et al. 2008a; Svensson et al. 2008b). In addition, TNFα modulates the release of glutamate in the central nervous system through activation of P55 receptors (Hermann et al. 2005; Youn et al. 2008). Increased interstitial concentrations of glutamate in the masseter muscle have been shown to induce a long lasting mechanical sensitization of masseter muscle nociceptors through activation of peripheral N-methyl-d-aspartate (NMDA) receptors (Cairns et al. 2007; Cairns et al. 2002; Mann et al. 2006). Finally, although TNFα stimulates the synthesis and release of CGRP in the trigeminal ganglion through activation of P55 receptors (Bowen et al. 2006), there is only indirect evidence for a role of CGRP in mechanical sensitization of the masseter muscle nociceptors at present (Ambalavanar et al. 2006). The aforementioned studies support the concept that TNFα may act indirectly to induce mechanical sensitization through a P55 and/or P75 receptor-mediated increase of various sensitizing substances in the masseter muscle.

Tissue accumulation of Evans blue dye is an indication of plasma protein extravasation (Fiorentino et al. 1999). In the present study, no difference in plasma protein extravasation between the TNFα and vehicle injected muscles was found, which suggests that intramuscular injection of TNFα does not induce plasma protein extravasation and thus is not grossly inflammatory. However, while the amount of Evans blue dye found in the masseter muscle of the vehicle group was substantially lower than that produced in the present study by formalin, or in a previous study by mustard oil (Fiorentino et al. 1999), it was about twice as high as the
The concentration of Evans blue dye previously found in the masseter muscle after a similar vehicle injection (2.2 μg/g) (Mann et al. 2006). The increased Evans blue tissue concentrations in the present study are consistent with our speculation of modest tissue trauma after vehicle injection. The masseter muscles are a common site of pain in patients suffering from either fibromyalgia or myofascial TMD (Dworkin and LeResche 1992; Hedenberg-Magnusson et al. 1997; Leblebici et al. 2007). Plasma and serum levels of TNFα are elevated in certain fibromyalgia patients (Bazzichi et al. 2007; Wang et al. 2008) and there is some evidence that TNFα levels are also elevated at myofascial trigger points (Shah et al. 2008). Although the exact pathophysiology of trigger points remains speculative, increased mechanical sensitivity is a characteristic feature of these myofascial trigger points. The present study suggests that when TNFα levels in skeletal muscle are elevated, mechanical sensitization occurs. Together, these findings indicate a potential role for TNFα in the pathophysiology of fibromyalgia and myofascial TMD. Future experiments to study the effect of TNFα on human muscle pain will be required to further bolster this concept.

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Figure legends

Figure 1. (A) The arrows on the photographs indicate examples of masseter muscle ganglion neurons positive for Fast blue (left) and P55 or P75 receptor-like immunoreactivity (right). (B) The graph indicates the frequency (%) of P75 and P55 receptor labelling in masseter ganglion neurons of different sizes. Although the distribution of P55 or P75 receptor-like immunoreactivity was similar in small, medium and large neurons, twice as many masseter ganglion neurons expressed the P75 receptor as expressed the P55 receptor.

Figure 2. (A) An example of a collision between an orthodromic and antidromic action potential is shown. The collision (*) indicated that this masseter afferent fiber (CV=8.5 m/s) projected to the caudal brain stem. (B) The tracings illustrate examples of afferent fiber (CV= 4.3 m/s) MT assessment before (baseline) and at 3 hours after the injection of 1 μg TNFα into the masseter muscle. The mean MT of this afferent fiber was 34 ± 5 before injection of TNFα, and 8.8 ± 1 g, three hours later. (C) The histogram shows discharge evoked by injection of HS into the mechanoreceptive field of the masseter muscle afferent fiber in (B). Responses to HS injection indicated that the recorded afferent fibers were putative nociceptors.

Figure 3. The line and scatter plot illustrates the effect of intramuscular injection of TNFα (0.1 μg, 1 μg) on the mean relative MT of rat masseter muscle nociceptors at various time points. Overall, the MT of nociceptors was significantly decreased by 1 μg TNFα but not by 0.1 μg TNFα or vehicle control (P < 0.05, two-way repeated measures ANOVA). The error bars
indicate standard error of mean. *:(p <0.05 compared to vehicle control, two-way repeated measures ANOVA and Holm-Sidak test).

**Figure 4.** The bar graph shows Evans blue dye accumulation in the masseter muscle 3 hours after injection of TNFα (0.1μg, 1μg) or vehicle control (n =6 rats per group). The concentration of Evans blue dye in the TNFα groups was not significantly different from the concentration of Evans blue dye in the control group (P >0.05, one way ANOVA). As a positive control, 37% formalin was also injected into the masseter muscle (n =3 rats) and Evans blue dye accumulation assessed after 3 hours. A substantially greater concentration of Evans blue dye after formalin was found. The error bars represent the standard error of mean.

**Figure 5. (A)** The Western blot shows the example of labelling of P55 receptor with P55 receptor antibody at 55 kDa in rat masseter muscle. (B) The graph illustrates the saturation of P55 receptor binding to masseter muscle homogenate by increasing concentrations of P55 receptor antibody. P55 receptor antibody binding to the muscle homogenate appeared to be saturated at concentration of 1μg/ml.

**Figure 6. (A)** The line and scatter plot illustrates the effect of 1μg TNFα, vehicle control (PBS), co-injection of 1μg TNFα and P55 receptor antibody on the masseter muscle nociceptors. P55 receptor antibody attenuated the development of TNFα-induced nociceptor mechanical sensitization. (B) The line and scatter plot illustrates the effect of 1μg TNFα, vehicle control (PBS), co-injection of 1μg TNFα and P75 receptor antibody on the masseter muscle nociceptors. Co-injection of TNFα and P75 receptor antibody significantly increased the mechanical threshold.
of nociceptors compared to 1µg TNFα alone or vehicle control. The error bars represent the standard error of mean. *: (p <0.05 compared to vehicle control, two-way repeated measures ANOVA and Holm-Sidak test).
Figure 1

(A)

Fast Blue

TNFα P55 receptor

(B)

Frequency (%)

Cell Area \( \mu \text{m}^2 \)

- Fast Blue
- TNFα P55 receptor

(B) Graph showing frequency distribution with bars for P75 and P55.
Figure 2 revised

(A) Orthodromic Antidromic

(B) Baseline MT MT 3 h after TNFα injection

(C) Firing rate (Hz) Time (s)

HS Injection
Figure 3

Relative MT (%)

Vehicle control
1 µg TNFα
0.1 µg TNFα

Time (min)

Baseline 60 120 180

0 50 100 150 200

0
Figure 4

Evans Blue dye Conc. (μg/g)

- Control
- 0.1 μg TNFα
- 1 μg TNFα
- Formalin
Figure 5

(A) A gel showing protein bands at 150 kDa and 55 kDa with an arrow indicating the P55 receptor antibody.

(B) Graph showing the percentage integrated density against concentration (μg/ml).

Concentration (μg/ml) vs. % Integrated Density:

- 0.00: 5
- 0.25: 10
- 0.50: 15
- 0.75: 20
- 1.00: 25
- 1.25: 25
- 1.50: 25
- 1.75: 25
- 2.00: 25
- 2.25: 25

Figure 5
(A) P55 receptor antibody + 1μg TNFα

(B) P75 receptor antibody + 1μg TNFα

Figure 6 revised