TNFα Mechanically Sensitizes Masseter Muscle Nociceptors by Increasing Prostaglandin E2 Levels.

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Abstract

Tumour necrosis factor alpha (TNF\(\alpha\)) induces mechanical sensitization of rat masseter muscle nociceptors which takes 2-3 hours to manifest and is mediated through activation of P55 and P75 receptors. The present study was undertaken to determine whether TNF\(\alpha\) induces nociceptor mechanical sensitization through the release of other algogenic substances such as glutamate, prostaglandin E2 (PGE2) and/or nerve growth factor (NGF), which have been shown to induce mechanical sensitization of muscle nociceptors. Masseter muscle homogenate levels of PGE2 and NGF were measured 3 hours after injection of TNF\(\alpha\) (1\(\mu\)g) or vehicle control using commercially available kits. Interstitial glutamate concentration was measured after injection of TNF\(\alpha\) or vehicle control using a glutamate selective biosensor probe. Diclofenac, a cyclooxygenase inhibitor that blocks the synthesis of PGE2, DL-2-amino-5-phophonovaleric acid (APV), a competitive NMDA receptor antagonist, and a tyrosine kinase A (TrkA) receptor antibody, which blocks NGF-induced masseter muscle nociceptor sensitization, were employed to assess the contribution of PGE2, glutamate, and NGF to TNF\(\alpha\)-induced nociceptor sensitization. PGE2 and glutamate concentrations were significantly elevated 3 hours after TNF\(\alpha\) injection into the masseter muscle. Injection of diclofenac partially reversed the TNF\(\alpha\)-induced decreases in the mechanical threshold (MT) of masseter muscle nociceptors, while vehicle control, APV and TrkA antibody did not significantly alter nociceptor MT. These results suggest that TNF\(\alpha\)-induced mechanical sensitization of masseter muscle nociceptors is mediated in part by increased PGE2 levels.

The findings of present study support the hypothesis that TNF\(\alpha\) induces a delayed mechanical sensitization of masseter muscle nociceptors indirectly by the release of PGE2.

Key Words;
Afferent fiber, Muscle pain, Mechanical threshold, Masseter muscle,Tumour necrosis factor alpha
Introduction

The mechanisms responsible for chronic muscle pain in conditions such as myofascial temporomandibular disorders and fibromyalgia are not known (Cairns 2010; Dworkin and LeResche 1992; Hedenberg-Magnusson et al. 1997; Leblebici et al. 2007). However, an association between elevated serum and plasma levels of tumour necrosis factor alpha (TNFα), a pro-inflammatory cytokine, and chronic muscle and joint pain has been previously reported (Bazzichi et al. 2007; Nordahl et al. 2000; Shah et al. 2008; Wang et al. 2008). In addition, many patients with chronic muscle pain have so-called myofascial “trigger points” which are areas of focal muscle hypertonicities that when palpated reproduce their muscle pain, and TNFα levels appear to be significantly elevated in these regions (Shah et al. 2008). It is thought that TNFα acts on P55 (TNFR1) and P75 (TNFR2) receptors to produce its pro-nociceptive effects in various tissues (Vandenabeele et al. 1995). Both P55 and P75 receptors are expressed by trigeminal ganglion neurons that innervate the masseter muscle as well as in the masseter muscle itself (Hakim et al. 2009). We have recently shown that intramuscular injection of TNFα causes a prolonged mechanical sensitization of masseter muscle nociceptors which takes 2-3 hours to manifest and is mediated via activation of peripheral P55 and P75 receptors (Hakim et al. 2009). However, it is not known whether some of the sensitizing effects of TNFα are mediated indirectly through the release of other nociceptive substances such as nerve growth factor (NGF), prostaglandin E2 (PGE2) and glutamate that have been shown to induce mechanical sensitization of muscle nociceptors (Cairns et al. 2002; Dong et al. 2009; Mense 1981; Murase et al. 2010; Svensson et al. 2010).

In other tissues, such as the skin, animal studies have shown that intraplantar injection of TNFα acts directly by exciting cutaneous afferent fibers (Junger and Sorkin 2000; Sorkin et al. 1997) as well as indirectly by inducing the release of other sensitising substances (Cunha et al. 1992, 2005; Russell et al. 2009; Woolf et al. 1997). Behavioural studies have shown that intramuscular injection of TNFα causes a delayed mechanical sensitization of skeletal muscle that appeared to be associated with
elevated levels of PGE2, NGF, and neuropeptides, although a cause-effect relationship was not
demonstrated in these studies (Schafers et al. 2003). In the central nervous system, TNFα also induces
release of glutamate through activation of P55 receptors (Hermann et al. 2005; Youn et al. 2008) and
enhances α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and N-methyl-D-aspartic acid
(NMDA) receptor currents as well as phosphorylation of the NR1 subunit of NMDA receptor (Wei et al.
2008). A 2-3 times increase muscle interstitial glutamate concentration results in significant
sensitization of masseter muscle nociceptors (Cairns et al. 2007). These results support the concept that
TNFα could be mediating sensitization of masseter muscle nociceptors by inducing release of other
algogenic substances.

The purpose of this study was to further explore the mechanism of TNFα-induced delayed
mechanical sensitization of masseter muscle nociceptors. It was hypothesized that TNFα acts indirectly
by inducing the release of NGF, PGE2 and/or glutamate and that these nociceptive mediators are
responsible for TNFα-induced masseter muscle sensitization. To test this hypothesis, glutamate
concentration was measured by using glutamate-selective biosensor probes, and NGF and PGE2 levels
were measured by Enzyme-linked immunosorbent assay (ELISA) after injection of a mechanically
sensitizing dose of TNFα (1μg). Electrophysiology experiments were performed to assess whether
these substances play a functional role in TNFα-induced mechanical sensitization of muscle nociceptors.

Diclofenac, a cyclooxygenase inhibitor that blocks synthesis of PGE2 (Cashman 1996; Gotzsche 2000),
DL-2-amino-5-phosphonovaleric acid (APV), a competitive NMDA receptor antagonist, and a tyrosine
kinase A (TrkA) receptor antibody, which blocks NGF-induced masseter muscle nociceptor
sensitization, were injected 3 hours after intramuscular injection of TNFα to determine whether these
antagonists could reverse TNFα-induced mechanical sensitization.
Methods

Animals. A total of 70 adult male Sprague-Dawley rats (300-400 g) 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.

Enzyme-linked Immunosorbent Assay. These experiments were done to determine masseter muscle concentration of PGE2 and NGF. TNFα (1μg) or vehicle control (n=5 in each group) was injected into rat masseter muscle and after 3 hours the rat was terminated with a high dose of pentobarbital (120 mg/kg). Approximately 1 cm² of masseter muscle tissue was harvested from around the injection site, which was marked on the overlying skin with a black marker. Muscle tissue was place on dry ice and stored at −70 °C. Tissues were weighed and homogenised using homogenization buffer (50mM Tris-Hcl PH 7.5, 150mM Nacl, 1% Triton, 0.1% Sodium Dodecyl sulphate, 0.5% Sodium deoxycholate). The average weight of muscle tissue in TNFα and PBS group was 0.20±0.03g and 0.25±0.05g respectively. Samples were centrifuged at 4 °C for 15 minutes at 15000 rmp. The supernatant of the homogenate was collected and muscle protein concentration was determined using Bradford method (Bradford 1976).

PGE2 concentrations. PGE2 level in muscle tissue homogenate was measured by enzyme immunoassay (Assay Design, Ann Arbor, USA) according to manufacturers’ instructions. The sensitivity of kit was 8.26 pg /ml. Samples and standards were run in duplicate and were averaged. The concentration of PGE2 was determined per gram of muscle protein.

NGF concentrations. NGF level in the muscle homogenate was measured by ELISA (Promega, Madison, USA) according to manufacturers’ instructions. The minimum sensitivity of kit was 7.8 pg/ml.
Both samples and standards were run in duplicate. The concentration of NGF was determined per mg of muscle protein.

**Glutamate concentration.** Glutamate biosensor probes (Pinnacle Technology Inc., USA) were used to measure interstitial glutamate concentration in the masseter muscle (Cairns et al. 2007). Glutamate probes were calibrated in-vitro according to the manufacturers’ instructions. The glutamate biosensor probe was inserted through a guide cannula, which was affixed to a catheter needle (3 mm between probe and needle tip), into the masseter muscle of isoflurane anesthetized adult male Sprague Dawley rats (n=10). Blood pressure, core body temperature, heart rate were continuously monitored throughout the experiment. After a 60-90 minute stabilisation period, the baseline glutamate concentration was measured over 10 minutes and subsequently TNFα or vehicle control (10μl, n=4) was injected into the masseter muscle near to glutamate biosensor probe. Masseter muscle glutamate concentration was measured each hour for 3 hours.

**Electrophysiological recording of muscle nociceptors.** Adult male Sprague Dawley rats (300-400g, n=45) were used for acute in vivo recording of extracellular action potentials from trigeminal ganglion neurons that innervate the masseter muscle (Cairns et al. 2002; Dong et al. 2007; Hakim et al. 2009). Isoflurane (2-2.5%) was used to anesthetise rats. A rectal thermometer was used to measure temperature. The trachea was cannulated and the rat was given artificial respiration using rodent ventilator. The carotid artery was cannulated to monitor blood pressure and to inject pentobarbital (100 mg/kg) to terminate the rat at the end of the experiment. 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 mmHg, 300–400 beat/min, and 60–80 mmHg, respectively. The rat’s head was placed into a streotaxic frame. The skin, muscle and dura overlying the caudal brainstem was reflected to allow stimulation of brainstem with stimulating electrode. A parylene-coated tungsten-recording electrode was
lowered into the trigeminal ganglion to record action potentials from single trigeminal ganglion neurons. A blunt probe was used to mechanically stimulate the masseter muscle as a search stimulus to identify trigeminal ganglion neurons whose afferent fibers had receptive fields within the masseter muscle (Cairns et al. 2001, 2002). The skin overlying the masseter muscle was pulled and pinch and pressure was applied to it to rule out innervation of the skin (Cairns et al. 2001, 2002). Masseter afferent fibers projecting to caudal brain stem were identified by the antidromic collision technique. Antidromic action potentials were evoked by stimulation of caudal brain stem with constant electric current. Masseter afferent fibers were mechanically stimulated to evoke orthodromic action potentials. Collision of antidromic and orthodromic action potentials was used to confirm the projection of masseter muscle afferent fibers to the caudal brainstem (Cairns et al. 2001, 2002). The conduction velocity of afferent fibers was calculated by dividing the distance between stimulating and recording electrodes by the latency of the antidromic action potential. To confirm that the mechanoreceptive field of each fiber was in masseter muscle, fibers were required to discharge in response to either injection of TNFα or hypertonic saline (HS; 1M), which was injected into the masseter muscle at the end of the experiment. Afferent fibers were excluded from further analysis if they failed to discharge in response to injection of at least one of the substances injected into the masseter muscle. The relatively high MT of most afferent fibers coupled to their response to HS, led us to identify these afferent fibers as putative nociceptors.

**Electrophysiology experiments.** These experiments were carried out to investigate the role of glutamate, NGF and PGE2 in TNFα-induced masseter muscle nociceptor sensitization. Nociceptors were assigned randomly to receive an injection of either vehicle control (10 µl phosphate buffered saline, n=10), the competitive NMDA receptor antagonist APV (10µl, 10mM or 50mM; n=10 per concentration), the non-steroidal anti-inflammatory drug (NSAID) diclofenac (0.1mg/ml, 10µl; n=7) or an antibody against the high affinity NGF TrkA receptor (2µg/ml, 10µl; n=8) 3 hours after TNFα injection. The investigator (AH) was unaware of the content of the injections. Baseline line mechanical
threshold (MT) of nociceptors was measured using an electronic von Frey hair (model 160IC, IITC) every minute for 10 minutes prior to insertion of the catheter needle that contained TNFα (Hakim et al. 2009). The baseline MT was determined by averaging 10 consecutive mechanical stimuli.

TNFα (Sigma; 1µg in 10µl phosphate buffered saline (PBS), n =10) was injected into the receptor field of the identified fiber and MT was measured after every hour for 10 minutes over 3 hours. Ongoing discharge was measured by counting the number of action potentials over 1 minute immediately before the baseline MT assessment, the injection of TNFα and each hour for 3 hours 1 minute before each MT assessment (Bove and Dilley 2010). Three hours after injection of TNFα, one of the test substances was injected and MT was measured once each minute for 30 minutes. At the end of MT recoding, HS was injected into the mechanoreceptive field of masseter nociceptor. Rats were then terminated by injection of pentobarbital.

Data analysis

The glutamate biosensor probe was connected to a 4 channel potentiostat (model 3104) and the input signal from the probe was analysed with pinnacle Acquisition Laboratory software (Pinnacle Technology Inc., USA). The change in glutamate concentration after injection of TNFα or PBS was determined by subtracting baseline glutamate concentration from the concentration of glutamate obtained after injection of TNFα or PBS.

The average nociceptor MTs at each time point was calculated. To account for the large variability in the baseline MT of individual fibers in each treatment group, relative MTs were calculated by dividing the mean MT at each time point by the mean baseline MT. This permitted us to compare changes in MT over time between treatment groups. To determine whether TNFα injections increased nociceceptor discharge, cumulative discharge was calculated by subtracting the number of action potentials recorded
for 10 minutes before injection from the number recorded for 10 minutes after injection. Change in ongoing discharge was calculated by subtracting the number of action potentials recorded before injection of TNFα from the number recorded after injection at each hour. Positive numbers indicated an increase in ongoing discharge, and negative numbers indicated a decrease in ongoing discharge.

Statistical analysis. The Pearson product moment test was used to determine whether correlations were significant. Two way repeated measures ANOVA and Holm-Sidak post hoc test were used to determine the differences in interstitial glutamate concentration over time after injection of TNFα and PBS. A Students t-test was used to compare mean levels of NGF between vehicle control and TNFα. One-way ANOVA was used to compare mean levels of PGE2 between TNFα, vehicle control and diclofenac injections. A logarithmic transformation of PGE2 concentrations was necessary to produce equal variance before running the ANOVA. The MTs after injection of various treatments (PBS, APV, NGF antibodies or diclofenac) were compared with the MT 3 hours after injection of TNFα through the use of one-way repeated measures ANOVA and Holm-Sidak post hoc test. Differences were considered statistically significant when p < 0.05. Unless otherwise indicated, data in the text are given as a mean ± standard error of the mean (SEM).
Results.

Effect of TNFα

In vivo single unit extracellular recording of 52 nociceptors was made. Seven nociceptors, which were not mechanically sensitized by TNFα three hours after injection, were excluded from further analysis.

The conduction velocity (CV) of the remaining 45 nociceptors was in the Aδ (CV=2-12m/s) range. The mean baseline MT of these nociceptors was 38 ± 5 g. HS was injected as positive control to confirm that the receptor field of the afferent fiber recorded was in masseter muscle and that all afferent fibers were nociceptors (Cairns et al. 2003b; Kumazawa and Mizumura 1977; Mense 1977; Paintal 1960).

TNFα evoked discharge in 12 of 45 nociceptors examined (median (interquartile range) cumulative discharge: 23(5-42) action potentials) (see example, Fig.1). Eight out of 45 nociceptors had ongoing activity prior to baseline MT assessment. Insertion of the needle into the masseter muscle caused 4 additional nociceptors to begin ongoing discharge, thus a total of 12 nociceptors exhibited some degree of ongoing activity (average 0.23 Hz, range 0.02-1.58 Hz) prior to TNFα injection. Three hours post injection, only 2 of the original 12 nociceptors having ongoing discharge had increased their discharge rate, while the rate of discharge had decreased or stopped altogether in the other 10 nociceptors.

However, 6 previously non-discharging nociceptors developed ongoing discharge by 3 hours post TNFα injection. Therefore, over the 3 hour period after injection, TNFα increased ongoing or induced novel ongoing discharge in a total of 8 of 45 nociceptors recorded.

Three hours after injection, TNFα (1μg) significantly decreased the MT of masseter muscle nociceptors as compared with the pre-injection baseline MT. The mean relative MT (%) of the 45 nociceptors that showed mechanical sensitization after 3 hours after TNFα injection was 39 ±3%. There was no significant correlation between baseline MT or CV and TNFα– induced mechanical sensitization.
Role of PGE2 in TNFα-induced Mechanical Sensitization

To investigate whether TNFα might be acting to increase prostaglandin levels or alter NMDA receptor activation, we tested the effect of the non-steroidal anti-inflammatory drug diclofenac on TNFα-induced mechanical sensitization at a concentration 0.1 mg/ml; a concentration which also competitively inhibits peripheral NMDA receptors (Dong et al. 2009) and prostaglandin synthesis (Cashman 1996; Gotzsche 2000). This concentration of diclofenac also inhibits prostaglandin synthesis, as demonstrated by our finding that TNFα injection significantly elevated levels of PGE2 as compared to vehicle control and that this TNFα-induced elevation in muscle PGE2 level was attenuated by diclofenac (Fig.2A).

Vehicle control injected 3 hours after TNFα injection had no significant effect on TNFα-induced mechanical sensitization at 10, 20 or 30 minutes post injection (Fig.2B). Diclofenac, however, when injected 3 hours after TNFα, partially reversed TNFα-induced mechanical sensitization of masseter muscle nociceptors. A significant effect of diclofenac on relative MT was seen at 10, 20 and 30 minutes after its injection (Fig.2C). These results suggested that an increase in PGE2 levels contributes to TNFα-induced mechanical sensitization, however, as the concentration of diclofenac employed also inhibits NMDA receptor activation, additional experiments were undertaken to examine the role of glutamate and peripheral NMDA receptor activation in TNFα-induced mechanical sensitization.

Role of Glutamate and Peripheral NMDA receptors in TNFα-induced Mechanical Sensitization

The mean baseline concentration of glutamate in the masseter muscle was 40 ± 10μM (n= 10). Two way repeated measures ANOVA revealed a significant effect of treatment (p<0.05) but not time on glutamate concentration, and there was no significant interaction between time and treatment (p=0.1). Post hoc tests revealed that TNFα significantly elevated the concentration of glutamate compared to vehicle control (Fig.3). However, as can be seen in Figure 3, the source of the difference between the
two treatment groups was principally due to a decline in interstitial glutamate concentration after injection of vehicle, rather than an increase in glutamate after injection of TNFα.

Injection of the competitive NMDA receptor antagonist APV (10mM or 50mM) 3 hours after injection of TNFα did not significantly change the MT of masseter muscle nociceptors at 10, 20 and 30 minutes after its injection (p<0.05 one way repeated measures ANOVA) (Fig.4 A,B). NMDA receptor activation did not appear to contribute significantly to the mechanism of TNFα-induced mechanical sensitization.

Role of NGF and TrkA receptors in TNFα-induced Mechanical Sensitization

TNFα injection did not significantly elevate the level of NGF as compared to vehicle control (Fig.5A). Injection of TrkA antibody 3 hours after TNFα injection did not significantly reverse TNFα-induced masseter muscle nociceptor sensitization at 10, 20 or 30 minutes after its injection (Fig. 5B). This result shows that NGF is not playing an important role in TNFα induced masseter muscle nociceptor sensitization.
Discussion

Injection of TNFα had no effect on ongoing discharge, but did produce mechanical sensitization of masseter muscle nociceptors that was associated with an increase in muscle concentrations of PGE2 and glutamate. Diclofenac, but not the NMDA receptor antagonist APV, partially reversed TNFα-induced sensitization of masseter muscle nociceptors. TrkA receptor antibody, which binds to the high affinity NGF receptor and has been previously demonstrated to attenuate masseter muscle nociceptor mechanical sensitization induced by exogenously administered NGF (Svensson et al. 2010) did not reverse TNFα induced mechanical sensitization of masseter muscle nociceptors. These results suggest that TNFα-induced sensitisation of masseter muscle nociceptors is mediated, at least in part, by PGE2.

PGE2 is a pro-nociceptive cytokine that sensitizes muscle nociceptors in both animals and humans (Dong et al. 2009; Mense 1981; Rukwied et al. 2007). In humans, PGE2 potentiates acid-induced muscle pain (Rukwied et al. 2007) and has been suggested to contribute to the development of masseter muscle pain in fibromyalgia patients (Hedenberg-Magnusson et al. 2001). Further, PGE2 in combination with bradykinin (BK), histamine and serotonin has a sensitizing effect on human muscle (Mork et al. 2003). In animals, PGE2 injection has been demonstrated to cause mechanical sensitization of rat gastrocnemius and masseter muscle nociceptors (Dina et al. 2008; Dong et al. 2009) and enhances the sensitizing response of BK on cat gastrocnemius muscle nociceptors (Mense 1981). The concentration of PGE2 measured in rat masseter muscle in the present study after vehicle control is similar to that previously reported for human skeletal muscle (Trappe et al. 2001). These findings indicate that an increase in the concentration of PGE2, for example due to tissue injury, contributes to the development of muscle pain through nociceptor sensitization (Graven-Nielsen and Mense 2001; Mense 1981; Tegeder et al. 2002). PGE2 induced sensitisation is mediated by PGE2 receptors EP2 and EP3 (Lin et al. 2006). These receptors are expressed on muscle tissue and trigeminal ganglion neurons (Graven-Nielsen and Mense 2001; Patwardhan et al. 2008). Studies have shown that TNFα treatment of
dorsal root ganglion cell culture or injection into rat gastrocnemius muscle significantly elevated the level of PGE2 (Fehrenbacher et al. 2005; Schafers et al. 2003). Our results are consistent with previous cutaneous animal experiments that have demonstrated that TNFα-induced sensitization is mediated through prostaglandins (Cunha et al. 2005; Russell et al. 2009).

We have recently shown that TNFα induces delayed mechanical sensitization of masseter nociceptors via activation of peripheral P55 and P75 receptors (Hakim et al. 2009). Activation of either TNFα receptor (P55 or P75) stimulated the release of PGE2 in synovial and gingival fibroblasts (Butler et al. 1994; Taylor 1993). *In vitro*, activation of P55 and P75 receptors by TNFα leads to the activation of P38 MAPkinases which in turn activate phospholipase A2 to liberate arachidonic acid, the precursor for prostaglandin synthesis (Ji and Woolf 2001). In the current study, PGE2 concentration was significantly elevated 3 hours post TNFα injection and diclofenac reversed the elevated level of PGE2, which supports the concept that TNFα acts through P55/P75 receptor mechanisms to increase the synthesis of PGE2 *in vivo*. However, since diclofenac only partially attenuated the effect of TNFα, although it completely reversed the TNFα-induced increase in PGE2 levels, it is conceivable that other algogenic substances also contribute to TNFα-induced mechanical sensitization of masseter muscle nociceptors.

Glutamate is an excitatory amino acid that causes sensitization of rat masseter muscle nociceptors through activation of peripheral NMDA receptor (Cairns et al. 2003b, 2007). A 200-300% increase in interstitial glutamate concentration of rat masseter muscle from baseline concentration is required to induce sensitization of masseter muscle nociceptors (Cairns et al. 2007). In the present study, TNFα increased glutamate concentrations on average by about 6 μM (~15%), which suggests that the increase in glutamate concentration induced by TNFα was likely not great enough to contribute to mechanical sensitization, at least through activation of peripheral NMDA receptors. It should noted that the
significant difference in glutamate concentration between TNFα and PBS injections mostly reflected a decrease in glutamate concentration after PBS injection, which we suggest was a result of local dilution of glutamate concentrations by the injectate (Cairns et al. 2003a). Nevertheless, it is possible that these small increases in interstitial glutamate concentrations could have activated non-NMDA receptors such as metabotropic glutamate receptors (mGluRs) or AMPA-activated inotropic glutamate receptors (GluRs), which have been shown to contribute to the development of nociception after inflammatory injury (Bhave et al. 2001; Dogrul et al. 2000; Walker et al. 2001). mGluR5 protein is expressed in the masseter nerve and in trigeminal ganglion and activation of mGluR5 induces mechanical sensitivity in the masseter muscle (Lee and Ro 2007). In addition, both GluR1 and GluR2 subtypes are expressed in trigeminal ganglion neurons (Chun et al. 2008) and injection of AMPA into masseter muscle both excites and mechanically sensitizes masseter muscle nociceptors (Dong et al. 2009). Although there is a potential that these other glutamate receptors might have been activated by the TNFα-mediated increase in interstitial glutamate concentration, previous findings that glutamate-induced mechanical sensitization of masseter muscle nociceptors can be completely reversed by NMDA receptor antagonists (Cairns et al. 2007) suggest that mechanisms other than activation of glutamate receptors are more important for TNFα-induced nociceptor sensitization.

NGF has also been shown to induce mechanical sensitization when injected into muscles (Andersen et al. 2008; Mann et al. 2006; Nie et al. 2009; Svensson et al. 2003, 2008a, 2010). Injection of NGF into the masseter muscle causes prolonged (~14 day) mechanical sensitization in healthy human subjects (Svensson et al. 2003, 2008a, b). In rats, the onset of mechanical sensitization of masseter muscle nociceptors by exogenously administered NGF into rat masseter muscle occurs within 30 minutes of injection and is mediated through activation of the TrkA receptors (Svensson et al. 2010). In the present study, TNFα (1μg) injection into the masseter muscle did not significantly elevate the level of NGF,
although in previous study TNFα injection into rat gastrocnemius muscle did significantly elevate the level of NGF as compared to vehicle control (Schafers et al. 2003). This discrepancy in results could be due to higher dose of TNFα (10μg) injected into rat gastrocnemius muscle in the Schafers et al. 2003 study. In addition, the concentration of NGF in our vehicle control treated masseter muscle was higher than baseline concentrations of NGF in other rat skeletal muscles (Wu et al. 2009), which may indicate that there are higher basal concentrations of NGF in the masseter muscle. These high baseline concentrations may have made it more difficult to detect a significant change in NGF levels by TNFα in the masseter muscle. However, even with this caveat, we also found no significant attenuation of TNFα-induced mechanical sensitization of masseter muscle nociceptors with the same concentration of a TrkA receptor antibody that we have previously demonstrated inhibits NGF-induced mechanical sensitization (Svensson et al. 2010). Taken together, these results suggest that NGF is also not playing important role in the mechanism of TNFα-induced mechanical sensitization of the masseter muscle.

TNFα modulates variety of ion channels. For example, TNFα enhances tetrodotoxin-resistant sodium currents; an effect which is mediated by the P38 mitogen activated protein kinase pathway through p55 receptor activation (Jin and Gereau 2006). TNFα has also been shown to reduce outward potassium currents in retinal ganglion neurons (Diem et al. 2001) and inhibits potassium currents in small dorsal root sensory neurons (Liu et al. 2008). Further, increases in PGE2 levels act to further enhance the inhibitory effects of TNFα on potassium currents (Liu et al. 2008). These results suggest that downstream modulation of sodium and/or potassium channel function by TNFα could also contribute to TNFα-induced mechanical sensitization of masseter muscle nociceptors. These additional mechanisms of nociceptor sensitization may explain why we were only able to partially attenuate TNFα-induced mechanical sensitization with diclofenac.
Clinical relevance:

Myofascial temporomandibular disorders are characterized by masticatory muscle pain and localized muscle tenderness, which some clinicians call “trigger points” but are more accurately described by the term “focal muscle hypertonicities” (Cairns 2010; Fricton 2007). Focal muscle hypertonicities are tender nodules within taut bands of skeletal muscle, which are painful upon palpation and refer pain to other body parts (Lavelle et al. 2007). Although, focal muscle hypertonicities are not associated with tissue damage or inflammation (Cairns 2010) elevated levels of a number of pro-inflammatory compounds such as bradykinin, adenosine tri-phosphate (ATP), substance P, calcitonin gene-related peptide (CGRP), neurotrophins, protons, interleukins (IL) and, importantly TNFα have been found in them (Shah et al. 2008), which suggests that some degree of tissue injury and/or inflammation could be occurring at these sites. Although, NSAIDS are a drug of choice for the treatment of myofascial Temporomandibular disorders-related pain conditions, in the small number of clinical studies undertaken to date, it has been difficult to demonstrate the efficacy of these agents for the treatment of muscle pain in these conditions (Fricton, 2007 #53; Cairns 2010). We previously found that TNFα mechanically sensitizes masseter muscle nociceptors without gross inflammation, and in the present study, that TNFα induces mechanical sensitization of muscle nociceptors that was partially attenuated by the NSAID diclofenac. We propose that injection of TNFα into skeletal muscles could be useful to model focal muscle hypertonicities and may prove useful to study mechanisms of NSAIDs for the treatment of masticatory muscle pain.

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Figure legends:

**Figure 1.** The histogram shows an example of TNFα evoked Aδ fiber (CV = 3.4 m/s, MT = 24g) masseter muscle afferent discharge. Baseline activity was recorded for 600 seconds and at 600 seconds; TNFα was injected into the mechanoreceptive field of masseter muscle afferent fiber, which evoked afferent fiber discharge.

**Figure 2.**

A. The bar graph shows the levels of PGE2 after injection of phosphate buffered saline (vehicle control), TNFα and TNFα with diclofenac (0.1 mg/ml). Injection of TNFα significantly elevated the levels of PGE2 as compared to vehicle control (n=5). Injection of diclofenac at 3 hours after TNFα injection significantly decreased the levels of PGE2 (*: p < 0.05 one-way ANOVA, Holm-Sidak post-hoc test).

B. The line and scatter plot illustrates the change in relative MT after injection of PBS at 10(T10), 20(T20) and 30(T30) minutes after its injection. TNFα significantly decreased the MT 3 hours after injection and this TNFα-induced decrease in MT was not significantly changed by injection of PBS.

C. The line and scatter plot illustrates the change in MT after injection of diclofenac (0.1mg/ml). TNFα significantly decreased the MT at 3 hours after injection and this decrease in MT was partially reversed by injection of diclofenac (*: p<0.05 one-way repeated measures ANOVA, n=7). The error bars indicate SEM.

**Figure 3.** The line and scatter plot shows the mean change in rat masseter muscle glutamate concentration at various time points after injection of TNFα (n= 4) or vehicle control (n= 4). Overall, the change in glutamate concentration after injection of TNFα was significantly higher than after injection of vehicle control. (*:p< 0.05 two way repeated measures ANOVA). The error bars indicate SEM.
Figure 4. The line and scatter plots illustrate the change in relative MT 10(T1), 20(T2) and 30(T3) minutes after injection of APV (A. 10mM, B. 50 mM). The TNFα-induced decrease in relative MT was not significantly altered by injection of either concentration of APV. The error bars indicate SEM.

Figure 5.A. Bar chart shows the level of NGF in the masseter muscle after injection of TNFα or vehicle control. Injection of TNFα did not significantly elevate NGF level compared to vehicle control (t-test, n=5). B. The line and scatter plot illustrates the change in MT 10(T1), 20(T2) and 30(T3) minutes after injection of TrkA antibody. TNFα significantly decreased the MT at 3 hours of its injection and this decrease in MT was not significantly reversed by injection of TrkA antibody (one way repeated measures ANOVA, n=8).
Fig. 1
Fig. 2.
Fig. 3.
Fig. 4
Fig. 5.