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THE ROLE OF TRPM2 IN HYDROGEN PEROXIDE-INDUCED EXPRESSION OF INFLAMMATORY CYTOKINE AND CHEMOKINE IN RAT TRIGEMINAL GANGLIA

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Abstract

Trigeminal ganglia (TG) contain neuronal cell bodies surrounded by satellite glial cells. Although peripheral injury is well known to induce changes in gene expression within sensory ganglia, detailed mechanisms whereby peripheral injury leads to gene expression within sensory ganglia are not completely understood. Reactive oxygen species (ROS) are an important modulator of hyperalgesia, but the role of ROS generated within sensory ganglia is unclear. Since ROS are known to affect transcription processes, ROS generated within sensory ganglia could directly influence gene expression and induce cellular changes at the soma level. In this study, we hypothesized that peripheral inflammation leads to cytokine and chemokine production and ROS generation within TG and that transient receptor potential melastatin (TRPM2), a well known oxidative sensor, contributes to ROS-induced gene regulation within TG. The masseter injection of complete Freund's adjuvant (CFA) resulted in a significantly elevated level of ROS within TG of the inflamed side with a concurrent increase in cytokine expression in TG. Treatment of TG cultures with H₂O₂ significantly up-regulated mRNA and protein levels of cytokine/chemokine such as interleukin 6 (IL-6) and chemokine (C-X-C motif) ligand 2 (CXCL2). TRPM2 was expressed in both neurons and nonneuronal cells in TG, and pretreatment of TG cultures with 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of TRPM2, or siRNA against TRPM2 attenuated H₂O₂-induced up-regulation of IL-6 and CXCL2. These results suggested that activation of TRPM2 could play an important role in the modulation of cytokine/chemokine expression within TG under oxidative stress and that such changes may contribute to amplification of nociceptive signals leading to pathological pain conditions.

Keywords

oxidative stress; myositis; IL-6; CXCL2; satellite glial cells

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CONFLICT OF INTEREST

There are no conflicts of interest associated with the present study.

INTRODUCTION

Trigeminal ganglia (TG) contain neuronal cell bodies of primary afferents that innervate oral and craniofacial structures. Each soma within TG is tightly surrounded by satellite glial cells (SGCs). It is well known that injury or inflammation of peripheral tissues leads to structural and functional changes in SGCs (Takeda et al., 2009). Activated glial cells produce pro-inflammatory factors such as cytokines, chemokines and neurotrophins. These soluble factors may modulate nearby neurons and possibly contribute to the development of hyperalgesia (Takeda et al., 2009). Injury or inflammation of craniofacial regions induces functional and morphological changes in both SGCs and neurons in TG (Garrett and Durham, 2008; Villa et al., 2010; Donegan et al., 2013). However, detailed mechanisms whereby peripheral injury or inflammation leads to the expression of cytokines and chemokines in cells within sensory ganglia are not clearly understood.

Reactive oxygen species (ROS) are reactive free radicals produced as byproducts of normal enzymatic reactions. It is well established that excess ROS generated in peripheral tissues and within the spinal cord following nerve injury or inflammation lead to pathological pain conditions (Gao et al., 2007; Wang et al., 2008; Kallenborn-Gerhardt et al., 2014). Recent studies indicate that ROS generated within sensory ganglia, such as dorsal root ganglia (DRG), regulate hypertension by altering the level of neuropeptides (Chapleau, 2007). Since ROS is known to have multiple functions including gene expression (Remacle et al., 1995), it is possible that ROS production within TG could lead to altered gene expression involved in nociceptive processing of craniofacial structures. However, it is not known whether ROS is actually generated within TG following tissue injury or inflammation.

Transient receptor potential melastatin 2 (TRPM2) is a member of the melastatin subfamily of TRP channels, which forms a calcium-permeable nonselective cationic channel. TRPM2 is expressed in neurons, microglia and immune cells and it is directly activated by intracellular ADP ribose and ROS including hydrogen peroxide (H_2O_2) (Sumoza-Toledo and Penner, 2011). A recent study suggested a functional role of TRPM2 in pathological pain (Haraguchi et al., 2012). TRPM2 activation in monocytes produces pro-inflammatory cytokines and induces infiltration of neutrophils, which contributes to aggravation of inflammatory responses (Haraguchi et al., 2012). Although neuronal expression of TRPM2 in sensory ganglia is suggested (Naziroglu et al., 2011a,b), it is unclear whether TRPM2 in sensory ganglia contributes to tissue injury-induced pathological changes within sensory ganglia. Since H_2O_2 is an endogenous agonist of TRPM2, it is possible that accumulation of ROS within TG following peripheral inflammation modulates cytokine/chemokine production within TG through TRPM2-dependent mechanisms.

The objectives of this project were to determine whether (1) masseter inflammation results in ROS and cytokine production in TG, (2) ROS, such as H_2O_2 , alters cytokine or chemokine expression in TG cultures and (3) TRPM2 expressed in TG is involved in the regulation of cytokine/chemokine gene expression.

EXPERIMENTAL PROCEDURES

Experimental animals

Adult male Sprague–Dawley (SD) rats were used in the present study. All animals were housed in a temperature-controlled room under a 12:12 light–dark cycle with access to food and water *ad libitum*. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and under a University of Maryland approved Institutional Animal Care and Use Committee protocol.

Assay of ROS in TG

ROS levels were quantified using a cell-permeant oxidant-sensing probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA; Invitrogen, Inc., Carlsbad, CA, USA). H₂DCFDA is de-esterified within cytoplasm and turns into highly fluorescent form upon oxidation. H₂DCFDA detects hydrogen peroxide, peroxy radicals, and peroxyxynitrite. H₂DCFDA has been successfully used not only in dissociated cells, but also in tissues (Behndig, 2008). Male SD rats (200 g) were injected with vehicle or 50 µl of 50% complete Freund's adjuvant (CFA) in isotonic saline into the masseter muscle. Naïve rats that did not receive either vehicle or CFA treatment served as a control group. Twenty-four hours after injection, the rats were anesthetized using sodium pentobarbital (100 mg/kg, i.p.), TG ipsilateral and contralateral to the injected muscle were quickly removed and washed with phosphate-buffered saline (PBS). Immediately after extraction and dissection, the tissues were minced finely in PBS and were incubated in 96-well plates in 200-µl PBS for 30 min at 37 °C. The background fluorescence for each specimen was determined with a fluorimeter (DTX880 Multimode Detector, Beckman Coulter) at 485 nm for excitation and 535 nm for emission. After the background reading, H₂DCFDA was added to each well to a final concentration of 10 µM. The plates were again incubated for 30 min at 37 °C, and the fluorescence was re-measured. ROS levels were estimated as the intensity of fluorescence after subtraction of the background fluorescence (Multimode Analysis Software). Negative control groups without TG tissues (PBS alone, PBS with H₂DCFDA, PBS with H₂O₂, PBS with H₂O₂ and H₂DCFDA) generated little or no positive signal which is only approximately 1% or less of signals obtained from TG tissues (Table 1). As a positive control, we examined whether addition of H₂O₂ to minced TG tissues can generate fluorescence signal in the presence of H₂DCFDA. TG samples with exogenously added H₂O₂ showed robust fluorescence signal in the presence of H₂DCFDA (Table 1). These control groups validate that our methods allow the detection of ROS in TG tissues by H₂DCFDA.

TG primary culture

TG were extracted from 200 to 250-g male SD rats and minced in DMEM/F12 (Sigma, St. Louis, MO, USA) containing fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA) and penicillin/streptomycin/glutamine (Gibco, Grand Island, NY, USA), on ice, incubated in media containing 1 mg/ml collagenase type IV (Sigma, St. Louis, MO) for 30 min at 37 °C with agitation. Following titration, cells were incubated for 15 min in 0.05% trypsin/0.1% EDTA (Gibco, Grand Island, NY) at 37 °C with agitation. Cells were cultured for 4 days before testing.

For the comparison of neuron-glia mixed culture and satellite glia-enriched culture, we used a modified culture protocol. Dissected TG tissues were treated with 0.1% collagenase D for 30 min. Then the tissues were further digested with trypsin (0.25%)–EDTA (0.02%) and DNase (type I, 50 µg/ml) in F12 medium at 37 °C for 15 min. The digested tissues were mechanically dissociated using polished pipettes. The cell suspension was centrifuged at 1000 rpm (170×g) for 1 min. After this step, we carefully collected both supernatant and pellet. First, the pellet was re-suspended in additional media. This fraction contains neurons as well as SGCs and thus called ‘neuron-SGCs mixed culture’. Second, the collected supernatant was centrifuged again at 1000 rpm for 1 min and the pellet was re-suspended in additional media. This fraction mainly contains SGCs rather than neurons and is called ‘SGCs-enriched culture’.

Western blot analysis

TG culture was lysed via a lysis buffer that included protease inhibitor cocktail. Lysate protein concentration was determined using Bio-rad protein assay. For each experiment, 10–20-µg total protein was loaded. Protein sample was denatured at 95 °C for 5 min and separated by 4–12% NuPAGE gel. Protein was blotted onto a polyvinylidene fluoride (PVDF) membrane. Membrane was blocked with 5% skim milk for 1 h at room temp. Membrane was incubated with antibody against TRPM2 (1:500; rabbit polyclonal, Novus Biologicals, Littleton, CO, USA), neuronal nuclei (NeuN) (1:5000; mouse monoclonal, Millipore, Temecula, CA, USA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:5000; mouse monoclonal, Calbiochem, San Diego, CA, USA). Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit (1:5000, Cell Signaling, Danvers, MA, USA) or goat anti-mouse (1:5000, Millipore) IgG antibodies. Bands were visualized with Amersham ECL Western blotting detection reagents (GE, Pittsburgh, PA, USA).

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Male SD rats (200 g) were injected with 50 µl of 50% CFA in isotonic saline into the masseter muscle. Naïve rats that did not receive either vehicle or CFA treatment served as control. Twenty-four hours after the injection, the rats were anesthetized using sodium pentobarbital (100 mg/kg, i.p.), TG ipsilateral to the injected muscle were quickly removed and washed with PBS, and prepared for RT-PCR experiment.

Total RNA was extracted from intact TG or cultured TG cells using Trizol (Sigma, St. Louis, MO) and purified with an RNeasy kit (Qiagen Sciences, Germantown, MD, USA) that included a DNase treatment to remove genomic DNA. Reverse transcription was carried out using the Superscript First Stand synthesis kit (Invitrogen, Carlsbad, CA). Superscript II (Invitrogen, Carlsbad, CA) was used to generate cDNA from 1 µg of RNA along with 2.5 ng of random primer per reaction. All primer pairs used in this study and average cycle numbers for each gene are described in Table 2. PCR amplification was performed with SYBR Green Supermix (Fermentas, Hanover, MD, USA) in a PCR program: 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s, 58 °C for 15 s, and 68 °C for 20 s in an Realplex real-time thermocycler (Eppendorf, New York, NY, USA). mRNA expression levels were analyzed by relative quantification methods. The amount of a given mRNA was

normalized to the GAPDH mRNA in the same sample and relative quantification of the mRNA of interest was calculated by the comparative CT method ($\Delta\Delta$ CT method) between groups.

siRNA

To knockdown the expression of TRPM2, we transfected the dissociated TG culture with a negative control siRNA (Silencer[®], Life Technologies) or siRNA specifically targeting rat TRPM2 5'-UAAGCGUUCAUGCUCUUC UGCCAGC-3' (500 pmol). The oligonucleotides were mixed with Lipofectamine[®] RNAiMAX Transfection Reagent (Life Technologies, Grand Island, NY, USA) according to the manufacturer's protocol and added to the cells. After an overnight incubation, the cells were exposed to H₂O₂ (100 μ M) for one hour followed by medium replacement and incubation without H₂O₂ for five hours. The cells were harvested for RNA and protein analyses.

Enzyme-linked immunosorbent assay (ELISA)

Interleukin-1 beta (IL-1 β), tumor necrosis factor alpha (TNF- α), interleukin 6 (IL-6), and chemokine (C-X-C motif) ligand 2 (CXCL2) were measured by two-antibody ELISA using biotin–streptavidin–peroxidase detection (University of Maryland Cytokine Core Lab). Polystyrene plates (Maxisorb; Nunc) were incubated with 50 μ l of homogenates from dissociated TG samples or standards prepared in assay buffer. Absorbance A₄₅₀ (minus A₆₅₀) was read on a microplate reader (Molecular Dynamics) and data analyzed using a SoftPro computer program (Molecular Dynamics).

Data analysis

Unless otherwise indicated, statistical comparisons of two independent groups were made with either Student's *t*-test or Mann–Whitney Rank Sum test. Data are presented as mean $\pm$ SE and differences were considered significant at $p < 0.05$.

RESULTS

To examine whether inflammation induces production of ROS in TG, we compared the intensity of fluorescence from ipsilateral TG obtained 1 day following the injection of CFA- or vehicle into the masseter muscle. To minimize experimental variations, samples from naïve or CFA- or vehicle-treated group were analyzed on the same day and the results from CFA- or vehicle-treated group were normalized to the results from naïve rats. Signals from ipsilateral TG of vehicle-injected rats were not significantly different from those in TG of naïve rats (Fig. 1). However, signals from ipsilateral TG of CFA-injected rats were significantly greater than those from TG of naïve rats. The ROS levels in contralateral TG from CFA-injected rats were not significantly different from those of naïve rats. These results suggest that masseter inflammation induces production of ROS in TG ipsilateral to the CFA injection.

Since ROS is known to modulate gene expression including pro-inflammatory cytokines (Lakshminarayanan et al., 1997; Jaramillo and Olivier, 2002; Chen et al., 2008; Yamamoto et al., 2008; Ma et al., 2009), we examined the level of transcripts of three representative

cytokines, IL-1 β , TNF- α and IL-6, and a chemokine CXCL2 in intact TG following CFA inflammation and in dissociated TG following direct application of H₂O₂. First, we assessed whether peripheral inflammation that leads to an increase in intraganglionic ROS is associated with up-regulation of cytokine/chemokine expression within TG. Significantly higher mRNA transcript levels were observed for the three cytokines, IL-1 β , TNF α and IL-6, in TG samples extracted 1 day after CFA treatment in the masseter muscle (Fig. 2A–C). There was also a tendency for up-regulation of CXCL2 transcript in TG from CFA-inflamed rats, but the difference did not reach statistical significance (Fig. 2D). The extent of increase was relatively moderate since the entire TG was assessed following the inflammation of masseter muscle. The average cycle number for the reference gene GAPDH, used for internal control, was stable between experimental conditions and did not change in response to H₂O₂ treatment.

In order to determine the direct effect of ROS on cytokine/chemokine transcription, H₂O₂ was applied to TG cultures at the concentration of 10 and 100 μ M for 1 h. Then the media were replaced with fresh media without H₂O₂ and incubated for an additional five hours before the cells were harvested. Such protocol minimizes cytotoxic effects of H₂O₂. Treatment of TG cultures with 10 μ M H₂O₂ induced a significant increase in mRNA levels for all four genes. The magnitude of mRNA up-regulation was relatively similar (between 2 and 4 folds) for all cytokines and chemokine analyzed (Fig. 2A–D). H₂O₂ at 100 μ M exerted differential effects on different cytokine genes. Significant increases in mRNA transcript levels compared to those of naïve samples were still observed for TNF- α , IL-6 and CXCL2 (Fig. 2E–H). H₂O₂ at 100 μ M led to lower levels of mRNA for IL-1 β and TNF- α than those produced by 10 μ M. However, the higher concentration of H₂O₂ led to further increase in mRNA levels for IL-6 and CXCL2. These results suggested that the optimal concentration of H₂O₂ for transcriptional up-regulation is different from cytokine to cytokine.

Increased synthesis of cytokine in TG culture following H₂O₂ treatment (100 μ M) was validated at the protein level by measuring the amount of cytokines/chemokine using ELISA. The magnitude of protein synthesis in TG samples was greatest for IL-6, followed by CXCL2, TNF- α , and IL-1 β (Fig. 3). Compared to vehicle control, the level of IL-6 protein increased by approximately 4-fold with 1-h treatment of H₂O₂ followed by medium replacement and an additional five hours of incubation (Control group, 530 $\pm$ 45 pg/ml; H₂O₂ group, 2002 $\pm$ 298; n =4 per group; p <0.01.) The level of CXCL2 protein was also significantly increased (Control group, 89 $\pm$ 9.8 pg/ml; H₂O₂ group, 155 $\pm$ 40.3; n =4 per group; p <0.01.) There was no significant difference in IL-1 β or TNF- α protein levels between vehicle and H₂O₂ treated groups. These results suggested that ROS differentially up-regulates pro-inflammatory cytokine/chemokine gene products in dissociated TG culture. Based on these results, we decided to focus on IL-6 and CXCL2 in subsequent experiments.

Since recent studies suggest that TRPM2 is involved in oxidative stress-induced up-regulation of cytokines (Sumoza-Toledo and Penner, 2011; Knowles et al., 2013), we examined whether TRPM2 mediates H₂O₂-induced up-regulation of cytokines in TG. TRPM2 mRNA transcript is reported to be expressed in TG (Vandewauw et al., 2013), but TRPM2 protein expression has not been demonstrated in TG. In western blot analysis using a specific antibody against TRPM2, we detected a band at approximately 190 kDa. This

band disappeared by pre-incubation of antibody with a control peptide (Fig. 4A). Furthermore, the density of the band was decreased by treatment of the TG culture with siRNA (500 ng) against TRPM2, but not by mismatch scramble sequence, further confirming the specificity of the primary antibody (Fig. 4B). The treatment with siRNA against TRPM2 down-regulated TRPM2 expression by approximately 60% ($p<0.05$). We also attempted to demonstrate TRPM2 protein localization in TG with immunohistochemistry, but the antibody used for staining TRPM2 in CNS neurons (Novus Biologicals, NB110-81601; (Chung et al., 2011) yielded non-specific staining in TG in our hands and we have yet to identify a reliable TRPM2 antibody for immunostaining.

As an additional attempt to examine the expression of TRPM2 in TG neurons and non-neuronal cells, we examined the expression of TRPM2 protein with Western blot in satellite glia-enriched culture condition (Fig. 5). When neuron-SGCs mixed culture samples and SGCs-enriched culture samples were blotted side by side, TRPM2 was detected both in mixed and SGCs-enriched samples. However, when the blot was probed using an antibody against NeuN, positive signal was detected only in mixed culture samples but not in SGCs-enriched samples. These results suggest that TRPM2 is expressed not only in TG neurons but possibly also in satellite glia and other non-neuronal cells.

To examine functional involvement of TRPM2 in cytokine production in TG, we initially tested the effects of 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of TRPM2 (Togashi et al., 2008), on H₂O₂-induced cytokine and chemokine gene up-regulation (Fig. 6). TG cultures were pretreated with dimethyl sulfoxide (DMSO) (0.025%) or TRPM2 inhibitor 2-APB (50 μ M) for 1 h. Then, H₂O₂ (100 μ M) along with either vehicle or 2-APB was applied for 1 h followed by incubation for 5 h without H₂O₂. In the presence of DMSO, H₂O₂ robustly up-regulated IL-6 and CXCL2 genes. In contrast, the increases were significantly, but not completely, suppressed in the presence of 2-APB. These results suggest the involvement of TRPM2 in the H₂O₂-induced increase in IL-6 and CXCL2 gene expression.

To further establish the contribution of TRPM2 in IL-6 and CXCL2 production, we tested the effects of knockdown of TRPM2 using siRNA (Fig. 7). We pretreated TG cultures with either control siRNA or TRPM2 siRNA overnight, which was followed by H₂O₂ treatment. Cytokine/GAPDH ratio for each sample was normalized to the value from TG samples treated with H₂O₂ only. IL-6 and CXCL2 up-regulation was significantly lower in groups treated with TRPM2 siRNA than negative control siRNA. These results demonstrated that TRPM2 contributes to H₂O₂-induced expression of IL-6 and CXCL2 in dissociated TG (see Fig. 8).

Since our data suggested the expression of TRPM2 both in TG neurons and satellite glia (Fig. 4), we examined whether H₂O₂ up-regulates IL-6 and CXCL2 gene in SGCs-enriched culture (Fig. 7). Treatment of SGCs-enriched culture with H₂O₂ significantly up-regulated IL-6 and CXCL2. These results suggested that ROS can regulate cytokine/chemokine genes in TG SGCs.

DISCUSSION

Excess ROS accumulated in a tissue can directly damage extracellular and cellular molecules and initiate cascades of cellular and molecular events that culminate in the development of pathological conditions. ROS generated in the spinal cord following nerve injury lead to sensitization of dorsal horn neurons via phosphorylation of N-methyl-D-aspartate (NMDA) and AMPA receptors (Gao et al., 2007; Kim et al., 2011) and through a loss of GABA_A neurons (Yowtak et al., 2013). ROS generated in the periphery are also critically involved in hyperalgesia following nerve injury or inflammation (Wang et al., 2008; Linley et al., 2012; Kallenborn-Gerhardt et al., 2014). However, there is limited information available on changes in ROS levels within either DRG or TG following peripheral injury. To address this, we adopted ROS-sensitive fluorescent dye procedure for detecting ROS generated within TG tissues *in vivo*. This approach provided evidence that masseter inflammation induces elevation of ROS levels within TG. Significant increase in ROS levels was observed on day 1 when CFA-induced mechanical muscular hyperalgesia is prominent (Niu et al., 2012). In the current study, we did not attempt to address mechanisms by which peripheral inflammation increases the level of ROS within TG, or which cell types ROS are derived from. We assume that pathological inputs from nociceptive terminals may remotely induce activities of enzymes involved in ROS generation or scavenging within TG. Indeed, oxidant and antioxidant enzymes are richly expressed in sensory ganglia (Ibi et al., 2008; Sato et al., 2013), implying that the level of ROS is tightly regulated within ganglia. Furthermore, oxidant and antioxidant genes are expressed both in SGCs and neurons (Sato et al., 2013), suggesting that both cell types may contribute to the ROS homeostasis within TG. Peripheral injury and inflammation can alter the expression or activity of these enzymes to shift the balance toward ROS accumulation within TG. In addition to the SGCs and TG neurons, the levels of inflammatory cytokines and chemokine can be further enhanced by resident macrophages and neutrophils attracted within TG during injury or inflammation (Jasmin et al., 2010; Villa et al., 2010). Therefore, identifying a key set of enzymes that undergo significant modulation during pathological conditions and determining cellular localization of the enzymes within TG should contribute to elucidating intraganglionic mechanisms that lead to pathological pain conditions.

Although the functional contribution of intraganglionic ROS should be determined, we presume that the increased level of intraganglionic ROS may contribute to masseter hyperalgesia. There is compelling evidence that ROS regulate the functions of primary afferents. Application of hydrogen peroxide to meninges induces electrical spiking activity of trigeminal nerves in a TRPA1-dependent manner (Shatillo et al., 2013). A ROS donor enhances excitatory synaptic transmission in rat spinal dorsal horn neurons, which is suppressed by antagonists against TRPA1 and TRPV1 (Nishio et al., 2013) suggesting activation or regulation of TRPA1 and TRPV1 channels by ROS in central terminals of primary afferent neurons. Depletion of glutathione, an antioxidant enzyme or administration of rotenone, a toxin used to generate intracellular oxidative stress, alters the ionic conductance in dissociated DRG neurons (Naziroglu et al., 2011a,b). Therefore, it is possible that the altered level of ROS within TG following muscle inflammation affects nociceptive molecules on the neuronal cell bodies within TG and thereby contributes to pain

conditions. In addition to these direct effects, it is possible that ROS affect nociceptors through indirect mechanisms. It is well known that oxygen-derived free radicals, such as H₂O₂, stimulate the synthesis of multiple inflammatory cytokines and chemokines in a variety of cells. H₂O₂ increases the production of IL-6 and IL-8 in airway epithelia (Chen et al., 2008), IL-1 β , IL-6, TNF- α , and TGF- β 1 in cardiac fibroblasts (Li et al., 2006), and TNF α in DRG cells (Ma et al., 2009). H₂O₂ also modulates chemokine expression in different cell types, including monocyte chemoattractant protein (MCP1) in epithelial cells (Lakshminarayanan et al., 1997), macrophage inflammatory protein-2 (CXCL2) in monocytes (Yamamoto et al., 2008), and MCP1 and macrophage-inflammatory protein-1 (MIP1) in macrophages (Jaramillo and Olivier, 2002). In the current study, H₂O₂ led to increased production of TNF α , IL-6 and CXCL2 in dissociated TG. Furthermore, experiments using SGC-enriched culture demonstrated that H₂O₂ could up-regulate cytokine and chemokine gene products in SGC. Thus, our data suggest that peripheral inflammation can induce significant up-regulation of inflammatory cytokines from neuronal and non-neuronal cells within TG via the generation of ROS. It is well established that pro-inflammatory cytokine (TNF α , IL-1 β , IL-6) production is regulated by a transcription factor, NF κ B (Schreck et al., 1991; Remacle et al., 1995). Since interactions of ROS and NF κ B are well known (Morgan and Liu, 2011), it is possible that H₂O₂ stimulates NF κ B activating pathways in TG.

The release and up-regulation of pro-inflammatory cytokines within sensory ganglia are considered hallmarks of functional interactions between sensory neurons and SGCs under pathological pain conditions (Takeda et al., 2009; Ji et al., 2013). It is possible that cytokines and chemokines are released from soma of primary afferents, and surrounding SGCs within TG and immune cells that are recruited to TG following masseter inflammation. IL-6 released from SGCs and neurons will likely affect nearby neurons or SGCs within TG (Brazda et al., 2013). Under injury condition, CXCL2 expressed by monocytes aids neutrophil recruitment into the ganglia (Stock et al., 2014). There is evidence that CXCL2 contributes to pathological pain conditions by aggravating peripheral and spinal pronociceptive inflammatory responses and that TRPM2 is required for CXCL2 expression under injury or inflammatory condition (Haraguchi et al., 2012). Another potential consequence of intraganglionic release of cytokines and chemokines could be the regulation of nociceptive genes in primary afferents. Sensory ganglia contain primary afferent cell bodies where transcriptional machineries are present. Signals generated by cytokines and chemokines in the immediate vicinity of transcription machineries are highly likely to affect transcriptional activities in nociceptors. A recent study suggests a close link between IL-6 and the expression of TRPA1 in DRG neurons (Malsch et al., 2014). Thus, increased production of ROS within TG can not only directly modulate nociceptor excitability at the cell body level, but also indirectly regulate nociceptive processing via regulation of inflammatory cytokines and chemokines.

In our study, we hypothesized that TRPM2 is a candidate molecule that mediates cytokine production during oxidative stress since H₂O₂ is an endogenous agonist of TRPM2 and TRPM2 activation has been shown to induce Ca²⁺-dependent synthesis of cytokines and chemokines (Sumoza-Toledo and Penner, 2011; Knowles et al., 2013). Attenuation of the increase in cytokine/chemokine gene expression following pharmacological inhibition of

TRPM2 by 2-APB or specific knockdown of TRPM2 using siRNA supports our hypothesis that TRPM2 contributes to H₂O₂-induced cytokine/chemokine expression. Despite dominant effects of TRPM2, we do not exclude a possible contribution of TRPM2-independent mechanisms since both pharmacological suppression and siRNA knockdown were incomplete. Although there are reports that TRPM2-like currents in DRG (Naziroglu et al., 2011a, 2013, 2014) and TRPM2 mRNA have been detected in TG (Vandewauw et al., 2013), expression of TRPM2 protein in sensory ganglia has not been shown. Our western blot analysis suggested that TRPM2 protein is expressed in neurons in TG. However, we acknowledge that our SGC-enriched TG cultures might also contain other cell types such as Schwann cells and macrophages (Glenn et al., 1993; Franceschini et al., 2012). Thus, our study suggests novel mechanisms involving TRPM2, a sensor of oxidative stress, by which peripheral inflammation leads to the production of cytokines and chemokines in both neurons and non-neuronal cells including SGCs that envelop neurons. Additional experiments are warranted to determine the relative contribution of TRPM2 in neuronal and non-neuronal cells. While the functional role of TRPM2 in TG needs to be tested, recent studies suggest that TRPM2 contributes to pathological pain conditions (Haraguchi et al., 2012; Isami et al., 2013). Knockout of TRPM2 in mice does not affect mechanical and thermal sensitivity but attenuates mechanical and thermal hyperalgesia in inflammatory and neuropathic pain models (Haraguchi et al., 2012). It was suggested that TRPM2 expressed in macrophage and microglia contributes to neuropathy and inflammation-induced hyperalgesia. Since these experiments were performed in conventional knockout animals lacking TRPM2 in all cell types (Haraguchi et al., 2012), it is possible that the function of TRPM2 expressed in primary afferents and SGC at least partially account for the attenuation of hyperalgesia in TRPM2-deficient mice.

CONCLUSION

We showed that TRPM2 contributes to the increased expression of cytokines/chemokine genes in TG under oxidative stress. Future studies addressing how oxidant-induced changes initiated within sensory ganglia affect primary afferent nociceptors should contribute to our understanding of the mechanisms underlying the development of dysfunctional pain states.

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Abbreviations

2-APB	2-aminoethoxydiphenyl borate
CFA	complete Freund's adjuvant
CXCL2	chemokine (C-X-C motif) ligand 2
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglia

EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
H₂DCFDA	2',7'-dichlorodihydrofluorescein diacetate
IL-1β	interleukin-1 beta
IL-6	interleukin 6
MCP1	monocyte chemoattractant protein
NeuN	neuronal nuclei
PBS	phosphate-buffered saline
ROS	reactive oxygen species
RT-PCR	real-time reverse transcription polymerase chain reaction
SD	Sprague Dawley
SGCs	satellite glial cells
TG	trigeminal ganglia
TNF-α	tumor necrosis factor alpha
TRPM2	transient receptor potential subfamily M member 2

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Fig. 1.

Increase in ROS within TG following masseter inflammation in rats. Relative intensity of fluorescence using H_2DCFDA , an indicator for ROS, of ipsilateral and contralateral TG obtained from naïve, CFA- or vehicle-injected rats. *Ipsilateral groups:* Naïve, CFA ($N=8$) and vehicle ($N=5$). Vehicle contralateral group ($N=3$). * $p < 0.05$, Student t -test.

Fig. 2.

Increase in cytokine/chemokine mRNA expression in TG. (A)–(D) RT-PCR analysis of IL-1β, TNF-α, IL-6, and CXCL2 from TG of naïve and CFA-inflamed rats. In CFA-treated rats, TG was analyzed one day after CFA administration in the masseter muscle. Relative changes in the cytokine/GAPDH ratio were calculated for each sample and the ratios of CFA-treated rats were normalized to those of naïve untreated rats. (E) and (F) RT-PCR analysis of IL-1β, TNF-α, IL-6, and CXCL2 from TG cultures following direct treatment of H₂O₂ (10 and 100 μM). Relative changes in the cytokine/GAPDH ratio were calculated for each sample and the ratios of experimental groups were normalized to the ratio of naïve sample. *N*=4 per group. **p*<0.05, Mann–Whitney Rank Sum test.

Fig. 3.

Increase in cytokine/chemokine protein expression in TG cultures. ELISA analysis of (A) IL-1 β , (B) TNF- α , (C) IL-6, and (D) CXCL2 from TG cultures treated with vehicle or H₂O₂ (100 μ M). The amount of protein detected in the samples was normalized to total protein.

$N=6$ per group. * $p<0.05$, Mann-Whitney Rank Sum test.

Fig. 4.

The expression of TRPM2 in rat TG culture. TRPM2 was detected in the lysates from rat TG cultures. (A) Western blot was performed using the antibody against TRPM2. As a negative control, the same protein sample was used for the pre-adsorption of the primary antibody against TRPM2 with an antigen peptide. (B) Rat-TRPM2 siRNA (500 pmol) was used to attenuate TRPM2 expression. Mismatch siRNA (MM) was used as a negative control.

Fig. 5.

Expression of TRPM2 in TG satellite glia-enriched culture. Western blot analysis of TRPM2 (upper), NeuN (middle) and GAPDH (lower) in neuron-SGC mixed cultures (Mixed) and satellite glia-enriched cultures (SG). TRPM2 was evaluated in four separate mixed (M1–M4) and SG (G1–G4) cultures. NeuN and GAPDH were evaluated in the same membrane. NeuN, a neuronal marker, was not detected in SG culture indicating a successful enrichment of SGCs.

Fig. 6. 2-Aminoethoxydiphenyl borate (2-APB) attenuates the effects of H₂O₂ on IL-6 and CXCL2 up-regulation. RT-PCR analysis of IL-6 (A) and CXCL2 (B) in H₂O₂-treated TG cultures with vehicle (DMSO) or 2-APB relative to naïve (dotted line). * $p < 0.05$ in Mann-Whitney U test. $N=7$ per group.

Fig. 7.

Effects of TRPM2 knockdown on IL-6 and CXCL2 expression. RT-PCR analysis of H₂O₂-induced up-regulation of IL-6 (A) and CXCL2 (B) in TG culture pretreated with a mismatch siRNA (MM) or siRNA against TRPM2. siRNA was treated overnight prior to the exposure to H₂O₂ (100 μ M). Fold changes in both treatment groups were normalized to those of H₂O₂ treatment group and plotted as percent changes. $N=8$ per group. * $p<0.05$ in Student's t -test.

Fig. 8. H₂O₂ up-regulates cytokine expression in satellite glia-enriched culture. RT-PCR analysis of H₂O₂-induced up-regulation of IL-6 (A) and CXCL2 (B) in satellite glia enriched cultures. *N*=5 per group. **p*<0.05 in Mann-Whitney Rank Sum test.

Table 1Validation of reactive oxygen species measurement from TG using H₂DCFDA

Groups	Intensity ^a	N
PBS	0.06 ± 0.09	5
PBS + H ₂ O ₂	-0.12 ± 0.05	5
PBS + H ₂ DCFDA	1.47 ± 0.38	5
PBS + H ₂ DCFDA + H ₂ O ₂	15.3 ± 4.5	5
Naïve TG + H ₂ DCFDA	1218 ± 279	5
Naïve TG + H ₂ DCFDA + H ₂ O ₂	21,757 ± 2605	5

^aBackground fluorescence was subtracted. Arbitrary unit.

Table 2

Sequences of primers for real-time PCR analysis and average Ct values

Gene	Primer sequences	Average Ct
CXCL2	Forward 5'-CCTACCAAGGGTTGACTTCAAGAA-3' Reverse 5'-GGCTTCAGGGTTGAGACAAACT-3'	30.20
IL-6	Forward 5'-CGAAAGTCAACTCCATCTGCC-3' Reverse 5'-GGCAACTGGCTGGAAGTCTCT-3'	28.65
TNF- α	Forward 5'-CCA GGA GAA AGT CAG CCT CCT-3' Reverse 5'-TCA TAC CAG GGC TTG AGC TCA-3'	26.50
IL-1 β	Forward 5'-CAC CTC TCA AGC AGA GCA CAG-3' Reverse 5'-GGG TTC CAT GGT GAA GTC AAC-3'	29.49
GAPDH	Forward 5'-TCACCACCATGGAGAAGGC-3' Reverse 5'-GCTAAGCAGTTGGTGGTGCA-3'	17.39