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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 H_2O_2 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 non-neuronal cells in TG, and pretreatment of TG cultures with 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of TRPM2, or siRNA against TRPM2 attenuated H_2O_2-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.

Key words: oxidative stress, myositis, IL-6, CXCL2, satellite glial cells.

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,

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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; H_2DCFDA, 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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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 (H_2DCFDA; Invitrogen, Inc., Carlsbad, CA, USA). H_2DCFDA is de-esterified within cytoplasm and turns into highly fluorescent form upon oxidation. H_2DCFDA detects hydrogen peroxide, peroxyl radicals, and peroxynitrite. H_2DCFDA 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, H_2DCFDA 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 H_2DCFDA, PBS with H_2O_2, PBS with H_2O_2 and H_2DCFDA) 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 H_2O_2 to minced TG tissues can generate fluorescence signal in the presence of H_2DCFDA. TG samples with exogenously added H_2O_2 showed robust fluorescence signal in the presence of H_2DCFDA (Table 1). These control groups validate that our methods allow the detection of ROS in TG tissues by H_2DCFDA.

Table 1. Validation of reactive oxygen species measurement from TG using H_2DCFDA

Groups	Intensity[a]	*N*
PBS	0.06 ± 0.09	5
PBS + H_2O_2	−0.12 ± 0.05	5
PBS + H_2DCFDA	1.47 ± 0.38	5
PBS + H_2DCFDA + H_2O_2	15.3 ± 4.5	5
Naïve TG + H_2DCFDA	1218 ± 279	5
Naïve TG + H_2DCFDA + H_2O_2	21,757 ± 2605	5

[a] Background fluorescence was subtracted. Arbitrary unit.

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'.