Neuroscience 248 (2013) 345–358

DIFFERENTIAL CELLULAR LOCALIZATION OF ANTIOXIDANT ENZYMES IN THE TRIGEMINAL GANGLION

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Abstract—Because of its high oxygen demands, neural tissue is predisposed to oxidative stress. Here, our aim was to clarify the cellular localization of antioxidant enzymes in the trigeminal ganglion. We found that the transcriptional factor Sox10 is localized exclusively in satellite glial cells (SGCs) in the adult trigeminal ganglion. The use of transgenic mice that express the fluorescent protein Venus under the *Sox10* promoter enabled us to distinguish between neurons and SGCs. Although both superoxide dismutases 1 and 2 were present in the neurons, only superoxide dismutase 1 was identified in SGCs. The enzymes relevant to hydrogen peroxide degradation displayed differential cellular localization, such that neurons were endowed with glutathione peroxidase 1 and thioredoxin 2, and catalase and thioredoxin 2 were present in SGCs. Our immunohistochemical finding showed that only SGCs were labeled by the oxidative damage marker 8-hydroxy-2′-deoxyguanosine, which indicates that the antioxidant systems of SGCs were less potent. The transient receptor potential vanilloid subfamily member 1 (TRPV1), the capsaicin receptor, is implicated in inflammatory hyperalgesia, and we demonstrated that topical capsaicin application causes short-lasting mechanical hyperalgesia in the face. Our cell-based assay revealed that TRPV1 agonist stimulation in the presence of TRPV1 overexpression caused reactive oxygen species-mediated caspase-3 activation. Moreover, capsaicin induced the cellular demise of primary TRPV1-positive trigeminal ganglion neurons in a dose-dependent manner, and this effect was inhibited by a free radical scavenger and a pancaspase inhibitor. This study delineates the localization of antioxidative stress-related enzymes in the trigeminal ganglion and reveals the importance of the pivotal role of reactive oxygen species in the TRPV1-mediated caspase-dependent cell death of trigeminal ganglion neurons. Therapeutic measures for antioxidative stress should be taken to prevent damage to trigeminal primary sensory neurons in inflammatory pain disorders.

Key words: trigeminal ganglion, glutathione peroxidase, catalase, transient receptor potential vanilloid subfamily member 1, caspase, inflammatory pain.

INTRODUCTION

Reactive oxygen species (ROS) are defined as oxygen-containing chemicals that are damaging to cellular components, such as lipids, proteins and nucleic acids. Oxidative stress stems from an imbalance in the generation and disposal of ROS, which leads to an elevated intracellular concentration of ROS and also leads to oxidative cell damage (Dringen et al., 2005). A superoxide anion ($O_2^{\cdot -}$) is generated, primarily by the mitochondrial respiratory chain and by cellular oxidases. The superoxide anion is quickly converted to hydrogen peroxide (H_2O_2) and oxygen by mitochondrial superoxide dismutase 1 (SOD1, Mn-SOD) and cytoplasmic SOD2 (Cu/Zn-SOD). H_2O_2 is degraded by several peroxide-decomposing enzyme systems, which include catalase, glutathione peroxidase (GPx) and the thioredoxin (Trx)/peroxiredoxin system (Salvemini et al., 2011). Of these systems, GPx1, a member of the GPx family, is known to play a major role in peroxide degradation in neural cells (Mitozo et al., 2011). Recent studies indicate that significant analgesic effects in both neuropathic pain (Khalil et al., 1999; Kim et al., 2004, 2010; Mao et al., 2009; Yowtak et al., 2011) and inflammatory pain (Thiemermann, 2003; Mika et al., 2007; Kuhad et al., 2008) are produced by the removal

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Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; ANOVA, analysis of variance; BAC, bacterial artificial chromosome; DMEM, Dulbecco's modified Eagle medium; EGFP, enhanced green fluorescent protein; flTRPV1, full-length TRPV1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLAST, glutamate/aspartate transporter; GPx, glutathione peroxidase; GST, glutathione S-transferase; HIV, human immunodeficiency virus; H_2O_2, hydrogen peroxide; HRP, horse radish peroxidase; JNK, c-Jun N-terminal kinase; $O_2^{\cdot -}$, superoxide anion; PBS, phosphate-buffered saline; Prx, peroxiredoxin; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SGCs, satellite glial cells; SOD, superoxide dismutase; STS, staurosporine; TBS-T, Tris-buffered saline/0.1% Tween-20; TEMPOL, 4-hydroxy-2,2,6,6-four methys piperidine; TG, trigeminal ganglion; TRPV1, transient receptor potential vanilloid subfamily member 1; Trx, thioredoxin.

0306-4522/13 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2013.06.010

of excess ROS by free radical scavengers. Either systemic or intrathecal administration of ROS scavengers assuaged hyperalgesia that was induced by a formalin injection (Hacimuftuoglu et al., 2006) and mechanical allodynia in neuropathic pain models (Khalil et al., 1999; Kim et al., 2004), implying that the site of algogenic ROS production was within the peripheral nervous system (Kim et al., 2004). The trigeminal ganglia (TG) harbor trigeminal primary neurons, and intraganglionic modifications of nociceptive signals have been described (Rappaport and Devor, 1994; Ceruti et al., 2011). In addition to primary sensory neurons, many specialized glial cells, termed satellite glial cells (SGCs), are present in TG (Hanani, 2010). Similar to TG neurons and Schwann cells, SGCs are derived from the neural crest (Maro et al., 2004). Morphologically, SGCs surround the neurons and form an envelope-like structure that ensheaths the neurons. Because of their small size and the relative paucity of specific markers, not much attention has been paid to these glial cells until recently. Nevertheless, evidence is now accumulating that shows that SGCs play important modulatory roles in the development of pain disorders (Takeda et al., 2009; Katagiri et al., 2012).

The capsaicin receptor, TRPV1 (transient receptor vanilloid subfamily, member 1), is a non-selective cation channel and serves as a transducer of various stimuli into pain signals in the nociceptors (Tominaga et al., 1998; Caterina et al., 2000; Khairatkar-Joshi and Szallasi, 2009). TRPV1 function is upregulated by inflammatory mediators using posttranslational mechanisms, which are critically implicated in the development of inflammatory hyperalgesia (Davis et al., 2000; Khairatkar-Joshi and Szallasi, 2009; Camprubi-Robles et al., 2009). In addition, excess TRPV1 activation has been shown to result in the activation of apoptosis-related cysteine proteases (called caspases) and cell death (Shin et al., 2003; Jin et al., 2005a; Amantini et al., 2007; Czaja et al., 2008).

In the present study, we show that Sox10, a transcription factor that is relevant to the development of neural crest-derived cells (Britsch et al., 2001), is a specific marker of SGCs in the adult TG and that antioxidant enzymes exhibit differential cellular localization in TG neurons and SGCs. Moreover, our cell-based studies demonstrate that excess TRPV1 activation is toxic to TRPV1-positive TG neurons because of ROS production and caspase-3 activation.

EXPERIMENTAL PROCEDURES

Animals

The *Sox10-Venus* bacterial artificial chromosome (BAC) transgenic mouse is described elsewhere (Shibata et al., 2010). Sox10 is a marker protein that is closely related to neural crest-lineage cells (Britsch et al., 2001). In the transgenic mouse, the fluorescent protein, Venus (Nagai et al., 2002), is expressed under the control of the *Sox10* promoter. As such, the generation of this transgenic mouse rendered the identification of Sox10-expressing cells feasible without immunostaining.

Male *Sox10-Venus* transgenic mice ($n = 8$; body weight, 20–25 g) were used for immunohistochemistry. Newborn rats ($n = 25$, postnatal day 2–3) were used for the preparation of primary TG cultures. All animal procedures were approved by the Laboratory Animal Care and Use Committee of our institution and were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of the Keio University School of Medicine (No. 08076). All surgeries and animal care were undertaken with the utmost caution to minimize the suffering of the animals.

Immunohistochemistry

Under deep anesthesia by excess pentobarbital sodium (Somnopentyl, Schering-Plough Animal Health Corp., Summit, NJ, USA), the animals were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0. Immediately after the perfusion fixation, TG were dissected out and immersed in the same fixative for 4 h at 4 °C and were then kept in 0.01 M phosphate-buffered saline (PBS) solution containing 20% sucrose (w/v) for 12 h for cryoprotection. Subsequently, the TGs were embedded in Tissue TEK (Sakura Finetek, Torrance, CA, USA) and were frozen in liquid nitrogen. Serial sections of 8-μm thickness were prepared on a cryostat (Reichert-Jung Cryocut 1800; Leica Microsystems, Mannheim, Germany) in the horizontal plane along the long axis. Every 15th section was thaw-mounted on MAS-GP micro slide glass (Matsunami, Osaka, Japan) and air-dried overnight at room temperature.

The sections were pre-incubated with 10% normal donkey serum/0.1 M phosphate buffer for 30 min for blocking. They were incubated with specific primary antibodies for 48 h at room temperature. After they were washed with 0.01 M PBS, the sections were incubated with species-specific fluorophore-labeled secondary antibodies for 2 h at room temperature. After rinsing with 0.01 M PBS, the sections were coverslipped in mounting medium (buffered glycerol: pH 8.6). The primary antibodies and dilutions used in the procedure were as follows: anti-glutamate/aspartate transporter (GLAST) polyclonal antibody (raised in guinea pig; code, AB1782; Millipore, Billerica, MA, USA; 1:200) (Benediktsson et al., 2012), anti-glutamine synthase monoclonal antibody (raised in mouse; code, MAB302; Millipore; 1:500) (Marin-Valencia et al., 2012), anti-glutathione S-transferase-pi (GST-π) polyclonal antibody (raised in rabbit; code, 311; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan; 1:200), anti-tubulin, βIII isoform (raised in mouse; code MAB1637; Millipore), anti-catalase polyclonal antibody (raised in rabbit; code, ab52477; Abcam, Cambridge, MA, USA; 1:200), anti-Trx2 polyclonal antibody (raised in rabbit; sc-50336; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:50) (McCommis et al., 2011), anti-SOD1 (Cu/Zn Enzyme) polyclonal antibody (raised in sheep; code, 574597; Calbiochem, Darmstadt, Germany; 1:200), anti-SOD2 (Mn Enzyme) polyclonal antibody (raised in sheep; code, 574596; Calbiochem, 1:200), and anti-GPx1 polyclonal antibody (raised in goat; code, AF3798; R&D