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EVALUATION OF MESENCHYMAL STEM CELL MODULATION OF TRIGEMINAL NEURONAL RESPONSES TO COLD

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Abstract—Tissue engineering protocols, such as regenerative endodontic procedures (REPs), comprise biologically based procedures designed to restore normal physiologic function. For REPs, the goal is reconstitution of the pulp-dentin complex by delivering mesenchymal stem cells (MSCs), including the stem cells of the apical papilla (SCAP) into a root canal system. Many patients regain cold sensitivity after REPs, but the mechanism is not understood. We hypothesized that SCAP modulate nociceptive function through a paracrine mechanism that activates cold-sensitive ion channels in neurons. We established a co-culture system with human SCAP and rat trigeminal (TG) sensory neurons in order to determine the effect of SCAP co-culture on neuronal responses using whole-cell patch-clamp electrophysiology. TG neurons co-cultured with SCAP demonstrated increased TRPA1-mediated ($p < 0.01$) and TRPM8-mediated inward current densities ($p < 0.01$) at 24 h in co-culture. Cold stimulation to SCAP significantly increased ATP release ($p < 0.01$), and supernatant collected after cold stimulation to SCAP was able to activate cultured TG neurons. Co-culture with SCAP significantly increased sustained ATP-evoked inward current density ($p < 0.05$). These data suggest that SCAP release trophic factors that act on afferent neurons to enhance cold-sensitive ion channel activity.

Keywords: mesenchymal stem cells, regenerative endodontics, TRPA1, TRPM8, ATP, apical papilla.

INTRODUCTION

Tissue engineering procedures are emerging across medicine and dentistry to restore the biological functions of injured tissues. In dentistry, regenerative endodontic procedures (REPs) have become a treatment alternative for immature teeth with open apices diagnosed with pulpal necrosis. These procedures rely heavily on the adequate disinfection of the root canal followed by recruitment of undifferentiated mesenchymal stem cells (MSCs) via evoked bleeding from adjacent apical dental papilla or bone (Lovelace et al., 2011; Chrepa et al., 2015).

Interestingly, positive responses to either cold or electrical stimuli have been reported in approximately half of patients after REPs (Diogenes et al., 2013, 2016). In normal healthy teeth, pulpal responses to cold stimuli depend on the inward movement of fluid within dentinal tubules and the subsequent activation of low-threshold A fibers that extend up to 300 μm into dentinal tubules (Narhi et al., 1984; Narhi, 1986; Ahlquist et al., 1994). This hydrodynamic theory of dentinal pain (Brannstrom et al., 1967; Brannstrom and Astrom, 1972; Trowbridge et al., 1980) postulates that the application of either cold or hyperosmotic solutions (Matthews and Vongsavan, 1994) onto the surface of a tooth triggers fluid movement and subsequent neuronal depolarization. However, this theory cannot explain cold sensitivity after REPs as a restorative material blocks neuronal access to coronal dentinal tubules (Diogenes et al., 2016). Thus, there is a gap in knowledge regarding mechanisms of cold transduction after REPs.

It is possible that cold detection may involve the direct activation of thermosensitive ionotropic channels expressed in free nerve ending of sensory neurons. Dental afferent neurons are contained within the trigeminal (TG) nerve and are known to express thermosensitive ion channels such as transient receptor potential ankyrin type 1 (TRPA1) and transient receptor potential melastatin 8 (TRPM8) (Nealen et al., 2003; Story et al., 2003; Kobayashi et al., 2005; Nagata et al., 2005; Kim et al., 2015). These channels are activated by temperatures below 17 °C and 25 °C, respectively and could be involved in the direct transduction of nociceptive signals upon cold stimulus (McKemy et al., 2002; Peier et al., 2002; Story et al., 2003; Kobayashi et al., 2005).

Further, it has been shown that adenosine triphosphate (ATP) can be released from odontoblasts upon cold stimulation (Shibukawa et al., 2015). This is a

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Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MSCs, mesenchymal stem cells; NGF, nerve growth factor; REPs, regenerative endodontic procedures; SCAP, stem cells of the apical papilla; TG, trigeminal; TRPA1, transient receptor potential ankyrin type 1; TRPM8, transient receptor potential melastatin 8.

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potentially significant observation since extracellular ATP may serve as a paracrine signaling molecule that activates neurons expressing purinergic receptors. Importantly, TG neurons express purinergic receptors, including the ionotropic channel P2X3 (Cook et al., 1997; Alavi et al., 2001; Renton et al., 2003; Staikopoulos et al., 2007; Kim et al., 2008). Together, the expression of TRPA1 and TRPM8 in TG neurons and the possible temperature-dependent release of ATP from dental pulp cells comprise two alternative hypotheses for cold-detection mechanisms after clinical REPs.

In this study, we evaluated whether a population of human MSCs isolated from the dental apical papilla (stem cells of the apical papilla (SCAP)) increase the activities of TRPA1, TRPM8 and P2X receptors in rat TG neurons. In addition, we determined whether SCAP releases ATP into the extracellular environment after a cold stimulus.

EXPERIMENTAL PROCEDURES

Tooth collection

This study was approved by the IRB of the University of Texas Health Science Center at San Antonio (UTHSCSA). Immature vital mandibular premolar teeth, with at least 2/3 root formation and an open apex, but with an indication for extraction for orthodontic treatment were collected after informed consent. The extracted teeth were immediately placed in ice-cold Hank's balanced salt solution (HBSS; Sigma Aldrich, St Louis, MO, USA) and used in temperature measurement experiments.

Ex vivo temperature measurement following simulated ''cold test'' in extracted human teeth

A thermocouple probe (0.4 mm in diameter) connected to an Oakton-360 thermometer (Oakton; Vernon Hills, IL, USA) unit was placed through the apical foramen of each tooth ($n = 6$) until reaching the pulp chamber roof. The positioning of the thermocouple probe was confirmed radiographically. Next, the teeth were placed in a 37 °C circulating water bath to the level of the cementoenamel junction to simulate the heat sinking properties of the periodontium. A cotton pellet (size 2) saturated with EndoIce™ (Hygenic; Akron, OH, USA) was placed directly onto the occlusal surface of each tooth for either 5 or 10 s. The change in the temperature was recorded for three consecutive tests 5 min apart. Next, in paired design, each tooth was accessed with a dental high speed handpiece fitted with a carbide bur (#1557, Brasseler; Savannah, GA, USA), the coronal pulp was removed with a round diamond bur to approximately 2 mm below the CEJ. Then, to simulate the typical restoration placed in REPs, a piece of collatape (Colla Tape®; Zimmer Dental; Carlsbad, CA, USA) was placed over the tissue, followed by a 3 mm of white MTA (Dentsply), a 3-mm layer of Fuji IX™, GC America, Alsip, IL, USA) and a final restoration with a bonded composite resin Z-100™, 3 M, St Paul, MN. The thermocouple was re-positioned in the coronal most aspect of the pulp space touching the pulp-restoration interphase and the temperature measurements repeated as described above.

SCAP

Previously characterized MSCs, namely stem cells of apical papilla (SCAP), were used in all experiments (Ruparel et al., 2013). Cells were cultured and expanded in media composed of alpha-minimum essential medium (Gibco, Grand Island, NY, USA) supplemented with heat-inactivated 10% fetal bovine, 1 L-glutamine (Gibco), penicillin (100 U/mL, Gemini, West Sacramento, CA, USA), and streptomycin (100 mg/mL, Gemini) to 10-cm cell culture dishes. Cells were allowed to expand in culture to 70–80% confluency followed by treatment with 0.05% trypsin (Gibco, Carlsbad, CA, USA). Cell suspension concentration was determined by a TC10 automated cell counter (Bio-Rad, Hercules, CA, USA), and cells between the third and eighth passages were used in all experiments in this study.

Rat primary TG neurons and SCAP co-culture

Adult male Sprague–Dawley rats (weight, 200–250 g each) (Charles River, Wilmington, MA, USA) were used in this study. All animal study protocols were approved by the IACUC of UTHSCSA and conformed to the International Association for the Study of Pain (IASP) and Federal guidelines. Animals were housed for 1 week before the experiments with food and water available *ad lib*. After isoflurane euthanasia, TG ganglia dissected from male rats were treated with collagenase-dispase (1 mg/mL; Roche), and neuronal cultures were prepared as previously described (Diogenes et al., 2006)

In brief, TG neurons were cultured onto 10-mm laminin-coated coverslips (1×10^3 neurons/coverslip) placed in 24 well plates, and allowed to attach for 2 h. Next, cell culture inserts fitted with 1-μm pore membranes (BD Biosciences, Bedford, MA, USA) containing 1×10^5 SCAP were placed in half of the wells containing the neurons. These inserts allowed for the co-culture of these two cell types and the exchange of soluble factors, while preventing their physical contact. The co-cultures were maintained in basal SCAP culture media (described above) at 37 °C and 5% CO_2 for 24 h. Nerve growth factor (NGF) was not added to neuron culture media since NGF has been reported to modulate TRPA1 and TRPM8 (Diogenes et al., 2007).

Patch-clamp electrophysiology

Recordings were made in whole-cell patch-clamp (holding potential (V_h) of −60 mV) configuration at 22–24 °C from the somata of small- to medium-sized neurons (15–40 pF). Data were acquired and analyzed using an Axopatch 200B amplifier and pCLAMP10.0 software (Axon Instruments; Union City, CA, USA). Recording data were filtered at 0.5–2.5 kHz and sampled at 2–10 kHz. Borosilicate pipettes with filaments (Sutter, Novato, CA, USA) were polished to resistances of 3–4 MΩ in whole-cell pipette solution. Access resistance