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Involvement of transient receptor potential melastatin-8 (TRPM8) in menthol-induced calcium entry, reactive oxygen species production and cell death in rheumatoid arthritis rat synovial fibroblasts

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ABSTRACT

Rheumatoid arthritis is most prominently characterized by synoviocyte hyperplasia which therefore serves as an important target for clinical therapy. In the present study, it was observed that menthol, the specific agonist of transient receptor potential melastatin subtype 8 (TRPM8), could induce sustained increases of cytosolic calcium concentration ($[Ca^{2+}]_c$) in synoviocytes isolated from collagen-induced arthritis rats in dose-dependent manner, which was evidently blocked by applying an extracellular Ca^{2+}-free buffer. Menthol-induced $[Ca^{2+}]_c$ increase was also significantly inhibited by potent TRPM8 antagonist capsazepine (CZP), indicating that this $[Ca^{2+}]_c$ elevation was mostly attributed to TRPM8-mediated Ca^{2+} entry. Besides, RT-PCR indeed demonstrated presence of TRPM8 in the synoviocytes. Meanwhile, it was found that menthol evoked production of intracellular reactive oxygen species, which could be abolished by Ca^{2+} free solutions or CZP. Further experiments showed that menthol reduced the cell numbers and survival of synoviocytes. This reduction was associated with apoptosis as suggested by mitochondrial membrane depolarization, nuclear condensation and a caspase 3/7 apoptotic assay. Menthol-induced death and apoptosis of synoviocytes both were obviously inhibited by CZP, intracellular calcium chelator BAPTA-AM, and reactive oxygen species inhibitor diphenylene iodonium, respectively. Taken together, our data indicated that menthol resulted in synoviocyte death associated with apoptosis via calcium entry and reactive oxygen species production depending on TRPM8 activation.

1. Introduction

Rheumatoid arthritis (RA) is an autoimmune and chronic disease characterized by inflammatory synovitis, leading eventually to cartilage and bone destruction, affecting over 50 millions people in the world (Firestein, 2003; McInnes and Schett, 2007). The etiology of this systemic inflammatory disorder is complex, and uncontrolled synoviocyte hyperplasia is an important feature in the genesis and development of RA (Bartok and Firestein, 2010). Relevant drug investigation and clinical application have been concentrated on the research of specific compounds which could lead to cell death or premature apoptosis in the affected RA tissue (Macpherson et al., 2006; Sherkheli et al., 2008).

Menthol ((1R, 2S, 5R)-2-isopropyl-5-methyl cyclohexanol) is a cyclic terpene alcohol produced by the peppermint herb, *Mentha piperita* (Al-Bayati, 2009; Patel et al., 2007). It elicits a pleasant cool sensation, and is widely used in food, cosmetics, and pharmaceutical products for treatment of asthma, headache, vertigo, and pain relieving (Eccles, 1994). As a natural compound with complex sensory effects, menthol exhibits multiple biological activities including anti-inflammation (Macpherson et al., 2006), antivirus (Al-Bayati, 2009), and antitumor (Kim et al., 2009; Wondergem and Bartley, 2009). Specially, it has been proposed that menthol acts on some membrane ion channels represented by transient receptor potential (TRP) channels (Sherkheli et al., 2010).

Transient receptor potential melastatin (TRPM) channels, the largest subfamily of TRP class, perform diverse functions ranging from detection of cold, osmolarity, redox state and cell proliferation or death (Clapham, 2003; Clapham et al., 2001; Zholos, 2010). TRPM8, a submember of TRPM, is predominantly expressed in sensory neurons, dorsal root ganglia, as well as prostate and other cancer cells (Dhaka et al., 2006; Journigan and Zaveri, 2013; McKemy et al., 2002; Yudin and Rohacs, 2012). It plays important roles in various pathological processes including pain sensation (Caspani et al., 2009; Levine and Alessandri-Haber, 2007; Proudfoot et al., 2006), inflammatory hyperalgesia (Sherkheli et al., 2010) and tumor migration (Yamamura et al., 2008).

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Accordingly, TRPM8 responds to cooling stimuli induced by cold temperatures or chemical agonists such as menthol and icilin (Zakharian et al., 2010). Upon the activation of TRPM8, extracellular calcium can enter into the cell via this Ca^{2+}-permeable channel, resulting in an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) (Peier et al., 2002; Thebault et al., 2005). This $[Ca^{2+}]_c$ increase eventually led to reduction of cellular viability in human melanoma (Yamamura et al., 2008), apoptosis in tumor cells like the prostate cancer-derived epithelial cell line (Park et al., 2009) and cell migration in human glioblastoma (Wondergem and Bartley, 2009). Studies also showed that TRPM8 mediated histamine release in mast cells (Cho et al., 2010), and analgesia in dorsal root ganglia neurons (Proudfoot et al., 2006).

Recently, the existence and expression of various types of TRP channels including TRPM8, TRPV1, TRPV4 have been detected in synovial fibroblasts from RA patients (Journigan and Zaveri, 2013; Biro et al., 2007; Kochukov et al., 2006). Our previous studies showed that TRPV1 and TRPV4 were implicated in the pathology of RA (Hu et al., 2008; Sun et al., 2008). However, the functions of other TRP channels in RA and underlying intracellular signaling remained largely unknown. In the present study, we examined the effects of menthol on $[Ca^{2+}]_c$, intracellular reactive oxygen species, mitochondrial membrane potential, caspase 3/7 activities, and cell survival in the arthritic rat synoviocytes, and investigated the roles of TRPM8 in these processes.

2. Materials and methods

2.1. Animals and reagents

Male Wistar rats weighing 200 ± 50 g were obtained from Institute of Health and Environmental Medicine, Academy of Military Medical Sciences (Tianjin, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, USA) and HyClone (Logan, UT, USA) respectively. Fura 2-AM was from Biotium (Hayward, CA, USA). RNAprep pure Cell/Bacteria Kit was from TIANGEN BIOTECH (Beijing, China). Reverse Transcription System, GoTaq PCR Core system and Caspase-Glo assay kit were from Promega (Madison, WI, USA). The rest of the reagents, including menthol, capsazepine (CZP), diphenylene iodonium (DPI), dihydroethidium (DHE), 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT), trypsin, Hoechst 33342, Rhodamine 123, EGTA, BAPTA-AM and collagenase II were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Isolation and culture of synovial fibroblasts

The collagen-induced arthritis rat models were established as reported (Shou et al., 2006), and the synovial fibroblasts were isolated and cultured as described (Hinoi et al., 2005). Briefly, rats with arthritis were sacrificed and their hind limbs were excised. Synovial membranes of the knee joints were carefully separated and minced in D-Hank's solution (NaCl 150 mM, KCl 5.4 mM, Na_2HPO_4 3 mM, KH_2PO_4 2 mM, Glucose 10 mM, pH = 7.4). Then, the synovial tissue was digested in 0.2% collagenase for 3 h at 37 °C in serum-free DMEM. After that, the cell suspension was centrifuged at 300*g* for 10 min and the isolated synovial cells were cultured in DMEM supplemented with 10% FBS in a humidified CO_2 incubator with 5% CO_2 at 37 °C. The cultured cells must be subjected to a minimum of 6 passages to obtain a pure culture for experiments as previously described.

2.3. Measurement of cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$)

Synoviocytes were incubated in Hanks' balanced salt solution (HBSS) (NaCl 150 mM, KCl 5.4 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, Glucose 10 mM and HEPES 10 mM, pH = 7.4) with 5 μM Fura 2-AM for 1 h at room temperature. After being washed gently and extensively with HBSS, cells were bathed in fresh HBSS. $[Ca^{2+}]_c$ was measured in a calcium imaging system built on an inverted fluorescence microscope (Olympus IX51). The ratiometric fluorescent Ca^{2+} indicator dye Fura 2 was alternately excited at 340 nm and 380 nm with a Lambda 10-2 optical filter changer (Sutter Instrument, USA). Fluorescence images (filtered at 515 nm ± 25 nm) were captured by a CCD camera (CoolSNAP fx-M, Roper Scientific Inc.) and quantitated with MetaFluor 5.0 (Universal Imaging Corporation, USA). All experiments were performed at room temperature in dark and $[Ca^{2+}]_c$ was represented by the ratio of fluorescence intensity at 340 nm/fluorescence intensity at 380 nm (F340/F380). At least three independent experiments were done for each condition and 5–10 individual cells were selected randomly in each experiment. One curve of calcium changes was plotted as the representation of other similar traces. Calcium-free HBSS was prepared by substituting $MgCl_2$ for $CaCl_2$ at the same concentration, with 2 mM EGTA added.

2.4. RNA isolation and RT-PCR

Total RNA from synoviocytes was isolated using RNAprep pure Cell/Bacteria Kit. Then, the RNA (1 μg) was subjected to reverse transcription using a reverse transcription system (Promega, USA) in a total volume of 20 μl reaction that contained $MgCl_2$ (5 mM), dNTP mixture (1 mM), oligo$(dT)_{15}$ primer (0.5 μg), RNase inhibitor (0.5 μl), reverse transcription 10 × buffer (2 μl), and AMV reverse transcriptase (15 U). The reaction mixtures were incubated at 42 °C for 30 min, 99 °C for 5 min to inactivate the enzyme, and then chilled on ice for 5 min. Subsequently, the product of RT reaction (1 μl) was amplified using a GoTaq PCR Core system (Promega, USA) in a total volume of 50 μl PCR buffer containing Green Master Mix (25 μl), sense primer (100 pM) and antisense primer (100 pM). The reaction mixtures were preheated to 95 °C for 2 min followed by 40 thermal cycles in a PCR machine (MJMini™, BIO-RAD, USA). For each cycle, denaturation was at 95 °C for 30 s, annealing at 61 °C for 30 s, and extension at 72 °C for 1 min. PCR primers were as follows: TRPM8 sense: 5′-ATCCCCATCGTGTGTTTTGC-3′, antisense: 5′-GAGGCGGACAAACTTGGGC-3′, encompassing 534 bp of the published rat TRPM8 sequence; GAPDH (glyceraldehyde-3-phosphate dehydrogenase, as positive control) sense: 5′-GTGGAGTCTACTGGCGTCTT-3′, antisense: 5′-CCAGGATGCCCTTTAGTG-3′, encompassing 537 bp.

2.5. Detection of intracellular reactive oxygen species

DHE, a reduced form of ethidium bromide, was used to detect and measure intracellular production of reactive oxygen species. After stimulation with indicated reagents for 1 h, synoviocytes were incubated in HBSS with 5 μM DHE for 30 min at 37 °C. The cells were then imaged after being rinsed twice. The excitation wavelength of DHE is at 488 nm and fluorescent images (after a 610 nm band-pass filter) were captured by CCD. MetaFluor was used to analyze the fluorescence intensity, which is indicative of the reactive oxygen species level.

2.6. Proliferation assay

The cells were plated in 24-well plates (1 ml; 1×10^5 cells/ml) at 37 °C in DMEM with 5% FBS, then trypsinized and counted three