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SHORT COMMUNICATION

Cold stress increases reactive oxygen species formation via TRPA1 activation in A549 cells

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Abstract Reactive oxygen species (ROS) are responsible for lung damage during inhalation of cold air. However, the mechanism of the ROS production induced by cold stress in the lung is still unclear. In this work, we measured the changes of ROS and the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) in A549 cell. We observed that cold stress (from 20 to 5 °C) exposure of A549 cell resulted in an increase of ROS and $[Ca^{2+}]_c$, which was completely attenuated by removing Ca^{2+} from medium. Further experiments showed that cold-sensing transient receptor potential subfamily member 1 (TRPA1) agonist (allyl isothiocyanate, AITC) increased the production of ROS and the level of $[Ca^{2+}]_c$ in A549 cell. Moreover, HC-030031, a TRPA1 selective antagonist, significantly inhibited the enhanced ROS and $[Ca^{2+}]_c$ induced by AITC or cold stimulation, respectively. Taken together, these data demonstrated that TRPA1 activation played an important role in the enhanced production of ROS induced by cold stress in A549 cell.

Keywords Cold stress · Reactive oxygen species · TRPA1 · $[Ca^{2+}]_c$

Introduction

Reactive oxygen species (ROS) is a universal and pleiotropic signaling molecule in the pathogenesis of disease states (Ray et al. 2012). In physiological states, ROS are normally kept at low levels, which makes very important for normal cellular function (Stowe and Camara 2009). However, the overproduction of ROS can cause serious damage to a variety of biomolecules (Belousov et al. 2006; Oliveira-Marques et al. 2009). In disease states, the dysregulated ROS signaling may contribute to be exclusively toxic to cells and tissues such as the lung (Finkel 2011). The production of ROS is provoked by a wide range of factors in the lung. These endogenous or exogenous mediators include cytokines and chemical irritants in polluted air (Thannickal and Fanburg 2000; Valavanidis et al. 2013).

As the interface with the outside air environment, the airway epithelial cell is critical to lung defense in response to exogenous stimulants. Cold temperature exposure can cause respiratory responses such as cough, bronchoconstriction, and mucosal secretion (Giesbrecht 1995; Koskela 2007). In addition, it has been reported cold-induced injury to lung epithelial cells is associated to ROS formation (Pizanis et al. 2011). A recent study showed that cold-sensing transient receptor potential subfamily member 1 (TRPA1) was expressed in the alveolar epithelial cells which increased release of IL-8 chemokine in response to activation of the TRPA1 (Mukhopadhyay et al. 2011). We have reported that cold stress enhances the production of nitric oxide through the activation of TRPA1 ion channel in A549 cells (Sun et al. 2014). However, the effect of TRPA1 activation on the production of ROS induced by cold stress in lung epithelial cells has not been studied in detail. In the present study, we investigated the role of TRPA1 in the production of ROS induced by cold stress using ROS and Ca^{2+} signaling fluorescence imaging.

Materials and methods

Reagents Fluo-2 acetoxymethyl ester (Fluo-2AM) was from Biotium Inc. (Hayward, CA, USA). Ethylene glycol-bis (β-amiROSethyl ether)-*N*,*N*,*N*,*N*-tetraacetic acid (EGTA), ROS

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probe dihydroethidium (DHE), TRPA1 antagonist HC-030031 and Calcium Ionophore A23187 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Allyl isothiocyanate (AITC) was obtained from Dewei chem (Chuzhou, China). The other agents were analysis grade.

Cell culture Human lung carcinoma A549 cells, a human alveolar epithelial cell line, were grown in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, 2 mM glutamine, 56 U/ml of penicillin-G, and 56 μg/ml of streptomycin sulfate. The cells were seeded on cover glass (24×24×0.17 mm) in six-well dishes (Costar, NY, USA) and were regulated at an initial density of 250,000 cells suspension in each well. Cells were located in a close chamber with a thermosensor and two pipes. The cold medium was rapidly infused into the chamber through one pipe and effused by another pipe. The change of solution temperature was recorded.

Solutions Cells were bathed in an isotonic medium (ISO). The medium contained 140 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 3.3 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 10 mM HEPES, and 5.5 mM glucose (pH 7.4, adjusted with NaOH, having an osmolarity of 300 mOsm/l). For studies in the absence of extracellular calcium ($[Ca^{2+}]_o$), the medium was made with $CaCl_2$ substituted by the same concentration of $MgCl_2$, with 1 mM EGTA to chelate trace Ca^{2+}.

Measurement intracellular ROS by DHE fluorescence A549 cells were washed with the above-mentioned isotonic medium buffer, incubated with 5 μM DHE in ISO buffer for 30 min at room temperature, and washed again with the buffer. Fluorescence (exCitation wavelength, 535 nm; emission wavelength, 610 nm) was measured using a fluorescence microscope (Olympus IX53, Tokyo. Japan). The cells' images were monitored through cool CCD (Q-imaging; Retiga EXI FAST 1394, Mono, 12-bit, cooled; Canada). Fluorescence images were collected at 60 min after stimulation. The ROS image experiments were repeated at least three times, and ten wells were used in each experiment. For quantization, the area of the cell was selected, and image quantization was performed using the Image J software (National Institutes of Health, USA). The background fluorescence was subtracted and the mean fluorescence intensity of the images was determined.

Measurement of $[Ca^{2+}]_c$ Fura 2-AM was dissolved in DMSO, with isotonic medium added to make a final concentration of 5 μmol/L. Cells were incubated for 1 h at room temperature in the dark and then were washed on the slip three times. Changes in the fluorescence intensity of Fura-2 at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm were monitored. The exposure time was 200 ms. Fluorescence intensity was measured through cool CCD and analyzed with Meta Fluor software (Sunnyvale, USA). F340/F380 was directly represented to $[Ca^{2+}]_c$. At least three experiments were carried out for each condition, and for each condition, 30 individual cells were selected. The representative calcium trace has been selected among more than 10 similar traces.

Immunohistochemistry We performed immunocytochemistry analyses in A549 cells. Cells were fixed with PFA for 30 min at room temperature. After three washing steps with PBS, cells were subjected to 0.1 % Triton-X 100 for cell membrane perforation. After treatment with protein block for 30 min, cells were stained with rabbit polyclonal TRPA1 antibody (1:250 Sigma, Saint Louis, USA) overnight at 4 °C. After three washings with TBS, FITC-conjugated goat anti-rabbit antibody (EarthOXford, San Francisco, CA, USA) were added and incubated for 90 min at room temperature. After removing excess of fluorescence-conjugated secondary antibody, cells were examined using a fluorescence microscope.

Statistical analysis All data were expressed as the means± standard deviation. Statistical analysis was assessed by Student's *t* test. In all cases, $p<0.05$ was considered significant; $p<0.01$ was considered statistically significant.

Results and discussion

The respiratory epithelial cells constantly interact with the external air environment. Cold air induces respiratory inflammation in high-altitude region or in winter (Koskela 2007). especially in patients with respiratory disorders (Seys et al. 2013; Hyrkäs et al. 2014). At addition, reactive oxygen species are involved in physiological and pathophysiological processes in the lung (Al Ghouleh et al. 2011). Lung epithelial cells have been identified in ROS production induced by various stimulants (Faux et al. 2009; Rosanna and Salvatore 2012). It has been reported that lung epithelial cells might increase ROS production after the cells were kept at 4 °C for varying periods in medium and then rewarmed (for 3 h) (Pizanis et al. 2011). However, the mechanism of the ROS production in pulmonary epithelial cells induced by rapid cold stress is still unclear.

Production of reactive oxygen species is dependent on $[Ca^{2+}]_c$ elevation induced by cold stimulation

We first examined the effect of cold stress on the ROS formation in A549 cells. The intensity of ROS in single cell was detected with ROS-specific fluorescence probe DHE. The change curve of medium temperature was shown in Fig. 1a (h). The ROS fluorescence images showed cold stress (from

20 to 5 °C) induced a substantial increase in A549 cells, which was significantly higher than control (Fig. 1a (a, b)). Statistical analysis of multicellular data confirmed that the enhanced production of ROS was induced by cold stimulation (Fig. 1b; $P<0.01$). In addition, we have observed the cellular viability with trypan blue. The results showed one-time cold stimulation did not bring damage to cell. These results showed that cold stress could increase the ROS formation in A549 cells.

The Ca^{2+} is an important signaling messenger that involves in the regulation of various cellular functions (Clapham 2007). It has been reported that ROS generated from mitochondrial electron transport chain which is modulated by mitochondrial Ca^{2+} uptake (Aldakkak et al. 2013). Furthermore, the mitochondria can accumulate a large amount of Ca^{2+} as a consequence of cytosolic Ca^{2+} increase (Rizzuto et al. 1999; Carafoli 2003). In addition, low temperature might increase ROS production in isolated mitochondria from murine brain (Ali et al. 2010). For the above reasons, the importance of $[Ca^{2+}]_c$ in the enhanced production of ROS induced by cold stress was further assessed in the following experiments. At first, the change of $[Ca^{2+}]_c$ has been examined during cold stimulation. The results showed cold stress (from 20 to 5 °C) induced a significant increase in $[Ca^{2+}]_c$ (Fig. 2a (b), Fig. 2b; $P<0.01$). In view of evidence that cold stress increases both $[Ca^{2+}]_c$ and ROS levels, the extracellular Ca^{2+} ions have been removed in the next experiment. The results showed Ca^{2+}-free medium almost completely blocked cold-induced $[Ca^{2+}]_c$ elevation (Fig. 2a (c)). At the same time, the increased production of ROS was also inhibited by cold stress without Ca^{2+} in the medium (Fig. 1a (c)). Statistical results confirmed the inhibitory effects of Ca^{2+}-free medium on the increase in $[Ca^{2+}]_c$ and ROS induced by cold stress in A549 cells (Figs. 1b and 2b; $P<0.01$, respectively). Next, the role of Ca^{2+} influx in the formation of ROS was studied with an alternative mechanism to increase $[Ca^{2+}]_c$. A23187, an ionophore for Ca^{2+}, has been used in the following experiments. The result showed A23187 induced robust elevation of both $[Ca^{2+}]_c$ and ROS in A549 (Figs. 1a (d) and 2a (d), Figs. 1b and 2b; $P<0.01$). These results indicate that $[Ca^{2+}]_c$ elevation from extracellular Ca^{2+} entry induced by cold stress plays an important role in the enhanced production of ROS in A549.

Production of reactive oxygen species is through the activation of TRPA1 during cold stimulation

In recent years, it has been demonstrated that transient receptor potential channel of the ankyrin-binding repeat subfamily, TRPA1, is the sole member of TRPA subgroup and a Ca^{2+}-permeable non-selective cation channel. TRPA1 could cause Ca^{2+} influx following activation by cold stress (<17 °C) or agonists (Nilius et al. 2012). Environmental chemicals have been identified to be TRPA1 activators such as allyl isothiocyanate (AITC) (Qian et al. 2013). The antagonist of TRPA1 is HC-030031 (Eid et al. 2008). It has been reported that cold temperature significantly increased mitochondrial ATP levels in cells which were transfected with cold-sensing transient receptor potential TRPM8 or TRPA1 (Park et al. 2013). However, the effect of TRPA1 channel on the production of ROS has not been reported. We hypothesized that $[Ca^{2+}]_c$ elevation following TRPA1 activated by the noxious cold is involved in the ROS production.

TRPA1 protein has been found in the airway epithelial cells toward the air in the human lung (Büch et al. 2013). The previous research has reported TRPA1 expression at mRNA and protein levels in A549 cell line (Mukhopadhyay et al. 2011). We also confirmed the conclusion of TRPA1 mRNA expression in A549 cell using RT-PCR in the previous research (Sun et al. 2014). Here, the expression of TRPA1 protein has been confirmed by immunocytochemistry in A549 cells (Fig. 1a (i)). In order to further investigate the possible role of TRPA1 channel in the production of ROS, A549 cells were treated with the TRPA1-specific agonist, allyl isothiocyanate (AITC), in our expirations. The results showed that AITC dramatically increased both $[Ca^{2+}]_c$ level (Fig. 2a (e), Fig. 2b, $P<0.01$) and ROS formation in A549 cells at room temperature (20 °C) (Fig. 1a (e), Fig. 1b; $P<0.01$). Next, HC-030031, a TRPA1-specific channel blocker, inhibited $[Ca^{2+}]_c$ elevation induced by AITC significantly (Fig. 2a (f), Fig. 2b; $P<0.01$). At the same time, HC-030031 also blocked the AITC-induced enhanced ROS production in A549 cells (Fig. 1a (g), Fig. 1b; $P<0.01$). Those results indicate that TRPA1 activation is involved in ROS formation in cells. In the following experiments, the results showed HC-030031 also largely prevented the cold-induced ROS and $[Ca^{2+}]_c$ elevation in A549 cells (Figs. 1a (j) and 2a (g)), respectively. Statistical results in Figs. 1b and 2b confirmed the suppressive effect of TRPA1 antagonist on the increase in ROS and $[Ca^{2+}]_c$ induced by cold stimulation. Taken together, those results suggest that $[Ca^{2+}]_c$ elevation via TRPA1 activation induced by cold stress is a possible mechanism underlying the enhanced production of ROS. Accumulating evidence has implicated that transient receptor potential canonical 3 is responsible for the increase in Ca^{2+}/calmodulin-dependent kinase II (CaMKII) activity and ROS production (Kitajima et al. 2011). Further study showed that mitochondrial ROS generation was mediated by Ca^{2+}/CaM/CaMKII signaling pathway (Toledo et al. 2014). We speculate that the increased ROS formation might be via Ca^{2+}/CaM/CaMKII signaling pathway which is elicited by TRPA1 activation induced by cold stimulation.

Reactive oxygen species (ROS) are well known to play a major role in the pathogenesis of a variety of lung disorders such as asthma, chronic obstructive lung disease, acute lung injury, pulmonary fibrosis, and cancer (Henricks and Nijkamp 2011; MacNee 2001). It has been reported that cold stress increased the production of ROS in animal model and cellular

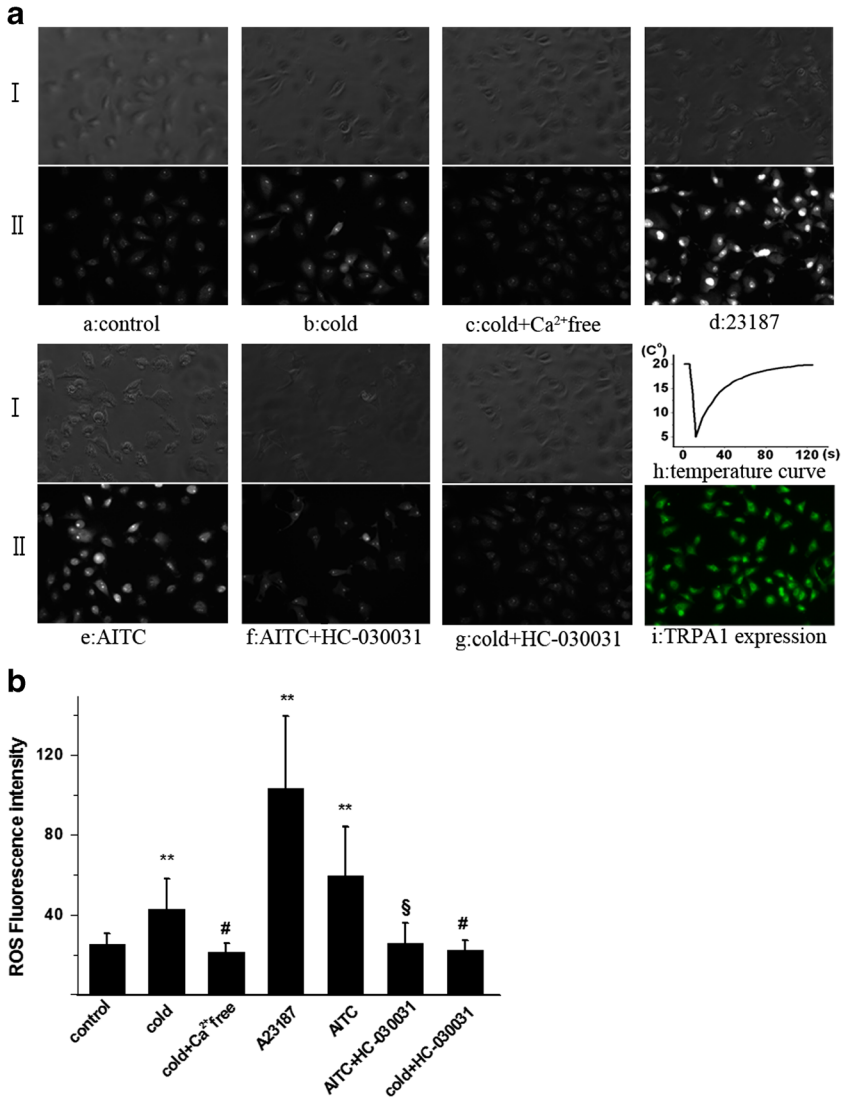

Fig. 1 The effect of stimuli with or without inhibitor on the ROS generation in A549 cells. **a** The fluorescence images of intracellular ROS produced in A549 cells. (*I*) A549 cells were imaged by interference contrast microscopy. (*II*) Fluorescence images are shown with the fluorescence intensity representing the ROS concentration. (*a*) Control. (*b*) Cold with 2 mM Ca^{2+} in the medium. (*c*) Cold and Ca^{2+} free in the medium. (*d*) 2 μM A32187. (*e*) 100 μM AITC. (*f*) 100 μM AITC and HC-030031 (50 μM pre-treatment for 20 min). (*g*) Cold and HC-030031 (50 μM pre-treatment for 20 min). (*h*) The change curve of temperature. (*i*) The protein expression of TRPA1 in A549. The immunocytochemistry revealed TRPA1 protein expression in A549. Cells were stained with rabbit TRPA1 antibody and FITC-conjugated goat anti-rabbit antibody. Immunocytochemistry experiments were carried out without primary antibody as control. No signals in cells were detected (control pictures no shown). **b** Statistical analysis of fluorescence intensity of ROS in A549 cells. Values are expressed as an averaged response (mean±S.E.M.) of at least 30 individual cells from three independent experiments. $^{**}P<0.01$, Cold, A23187 and AITC increased the production of ROS compared with control. $^{\S}P<0.01$; HC-030031 inhibited the production of ROS stimulated by AITC. $^{\#}P<0.01$; Ca free and HC-030031 inhibited the production of ROS stimulated by cold stress, respectively

and mitochondrial experiments, respectively (Ali et al. 2010; García-Díaz et al. 2015; Awad et al. 2013). Our results suggest that TRPA1 is mainly involved in the production of ROS via $[Ca^{2+}]_c$ elevation pathway induced by cold stress in lung epithelial cell. The pulmonary expression of TRPA1 has been found in sensory nerve endings and pulmonary epithelial cells, which is involved in the acceleration of inflammatory responses in the lung (Büch et al. 2013). On the other hand, ROS are important components of signaling cascades that respond to extracellular stimuli, including tumor necrosis factor, interleukin-1, adenosine-5′-triphosphate, cigarette smoke extract, lipoteichoic acid, or lipopolysaccharide, which are associated with many of the known inflammatory target proteins, such as metalloproteinase-9, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, cyclooxygenase-2, and cytosolic phospholipase A2. Noxious stimuli-induced ROS result in the modulation of inflammatory gene expression associated with pulmonary diseases (Lee and Yang

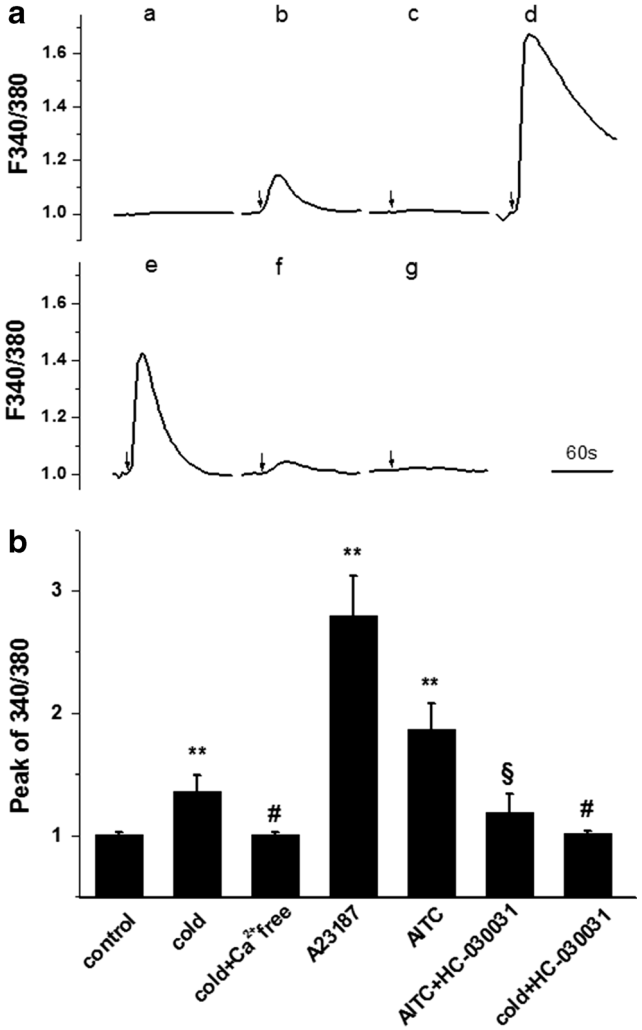

Fig. 2 The change of $[Ca^{2+}]_c$ in A549 induced by stimuli with or without inhibitor. **a** Representative tracings of intracellular Ca^{2+} fluorescence intensity in A549 cells. (*a*) Control: without change of $[Ca^{2+}]_c$. (*b*) Cold with 2 mM Ca^{2+} in the medium. (*c*) Cold with Ca^{2+}-free medium. (*d*) 2 μM A23187. (*e*) 100 μM AITC. (*f*) 100 μM AITC and HC-030031 (50 μM pre-treatment for 20 min). (*g*) Cold and HC-030031 (50 μM pre-treatment for 20 min). *Arrows* point to the beginning of cold stress or agonist treatment. **b** Statistical analysis of the peak of $[Ca^{2+}]_c$ in multiple experiments. Values are expressed as an averaged response (mean± S.E.M.) of at least 10 individual cells from three independent experiments. $^{**}P<0.01$; cold, A23187 and AITC increased the peak of $[Ca^{2+}]_c$ elevation compared with control, respectively. $^{\#}P<0.01$; Ca free and HC-030031 inhibited the peak of $[Ca^{2+}]_c$ elevation stimulated by cold stress, respectively. $^{\S}P<0.01$; HC-030031 blocked the peak of $[Ca^{2+}]_c$ elevation induced by AITC

2013). In addition, it is worth noting that TRPA1 channel exhibits a cooperative effect during cold and other chemical stimulation simultaneously (del Camino et al. 2011). Therefore, we speculate the ROS elevation which is dependent on TRPA1 activation aggravates the pulmonary disease during cold environment. Perhaps this work might improve the understanding of the pathological process of the pulmonary disease.

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