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Original Contribution

TRPM2 channels in alveolar epithelial cells mediate bleomycin-induced lung inflammation

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ABSTRACT

Lung inflammation is a major adverse effect of therapy with the antitumor drug bleomycin (BLM). Transient receptor potential melastatin 2 (TRPM2) is a Ca^{2+}-permeable channel that is activated by oxidative stress through the production of ADP-ribose. We herein investigated whether TRPM2 channels contributed to BLM-induced lung inflammation. The intratracheal instillation of BLM into wild-type (WT) mice increased the number of polymorphonuclear leukocytes (PMNs) and inflammatory cytokine levels in the lung. Increases in inflammatory markers in WT mice were markedly reduced in *trpm2* knockout (KO) mice, which demonstrated that the activation of TRPM2 channels was involved in BLM-induced lung inflammation. The expression of TRPM2 mRNA was observed in alveolar macrophages, alveolar epithelial cells, and lung fibroblasts. Actually, TRPM2 protein was expressed in lung tissues. Of these, TRPM2 channels in epithelial cells were activated by the addition of H_2O_2 following a BLM pretreatment, resulting in the secretion of macrophage inflammatory protein-2 (MIP-2). The H_2O_2-induced activation of TRPM2 by the BLM pretreatment was blocked by the poly(ADP-ribose) polymerase (PARP) inhibitors PJ34 and 3-aminobenzamide. The accumulation of poly(ADP-ribose) in the nucleus, a marker for ADP-ribose production, was strongly induced by H_2O_2 following the BLM pretreatment. Furthermore, administration of PRAP inhibitors into WT mice markedly reduced recruitment of inflammatory cells and MIP-2 secretion induced by BLM instillation. These results suggest that the induction of MIP-2 secretion through the activation of TRPM2 channels in alveolar epithelial cells is an important mechanism in BLM-induced lung inflammation, and the TRPM2 activation is likely to be mediated by ADP-ribose production via PARP pathway. TRPM2 channels may be new therapeutic target for BLM-induced lung inflammation.

1. Introduction

Transient receptor potential melastatin 2 (TRPM2) is a Ca^{2+}-permeable non-selective cation channel that is activated by oxidative stress such as H_2O_2 [21]. The expression of TRPM2 has been observed in the brain, lungs, and inflammatory cells including neutrophils and monocytes/macrophages [21,25,26,51]. Regarding the physiological role of TRPM2 channels, the activation of these channels by oxidative stress has been shown to cause intracellular Ca^{2+} overload, thereby mediating cell death in various types of cells [21,28–30]. In addition to cell death, we previously

Abbreviations: ADPR, ADP-ribose; AECs, alveolar epithelial cells; α-SMA, α-smooth muscle actin; 3-AB, 3-aminobenzamide; AMs, alveolar macrophages; BAL, bronchoalveolar lavage; BLM, bleomycin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1β, interleukin-1β; IL-8, interleukin-8; KC, keratinocyte-derived cytokine; LPS, lipopolysaccharide; MIP-2, macrophage inflammatory protein-2; PARG, poly(ADP) glycohydrolase; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; PMNs, polymorphonuclear leukocytes; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; TRPM2, transient receptor potential melastatin 2

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reported that H_2O_2-induced Ca^{2+} entry through TRPM2 channels stimulated the secretion of interleukin-8 (IL-8) in monocytes [51]. TRPM2-mediated Ca^{2+} entry has also been associated with lipopolysaccharide (LPS)-induced cytokine secretion in monocytes [50]. The findings of studies using *trpm2* knockout (KO) mice implicated TRPM2 channels in the development of various inflammatory diseases such as ulcerative colitis, myocardial ischemia/reperfusion injury, neuropathic pain, and autoimmune encephalomyelitis [22,26,35,51]. In contrast, especially in the lung, previous studies have suggested that TRPM2 does not play a role in the progression of inflammatory diseases. Hardaker et al. [23] found no significant differences in LPS-induced lung inflammation between WT and *trpm2* KO mice. Furthermore, Sumoza-Toledo et al. [45] showed that the severity of ovalbumin-induced airway inflammation was not reduced in *trpm2* KO mice.

Bleomycin (BLM) is a glycopeptide antibiotic with potent antitumor activity against a range of squamous cell carcinomas, testicular cancers, and malignant lymphomas [3]. The antitumor activity of BLM is considered to cause DNA damage through the production of oxygen radicals [6,11]. A major adverse effect of BLM is its induction of lung injury [8]. Lung toxicity develops in approximately 10% of patients receiving BLM, and may progress to pulmonary fibrosis in 1% of patients [8]. Although the underlying mechanisms have not yet been elucidated in detail, various factors have been suggested to play roles in BLM-induced lung inflammation including alveolar macrophages (AMs) [42,44], inflammatory cytokines [41,52], reactive oxygen species (ROS) [33,47,9], and poly(ADP-ribose) polymerase (PARP) [19,20,27]. ROS-induced TRPM2 channel activation is known to be mediated by intracellular ADP-ribose (ADPR) produced through the combined action of PARP and poly(ADP) glycohydrolase (PARG) [7,14]. These findings prompted us to hypothesize that the activation of TRPM2 channels may be involved in the development of BLM-induced lung inflammation.

In the present study, we determined whether the BLM-induced lung inflammation at early stage was ameliorated in *trpm2* KO mice, and demonstrated that BLM-induced, but not LPS-induced lung inflammation, was less severe in *trpm2* KO mice.

2. Materials and methods

2.1. Animals

The generation of *trpm2* KO mice has been described previously [51]. Male *trpm2* KO and littermate WT mice (C57BL/6J background, 6–10 weeks of age) were used in this study and handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The experimental protocol was approved by the Experimental Animal Committee of Showa University (#20080), Yokohama College of Pharmacy (#14–37), and Teikyo Heisei University (27051).

2.2. Experimental model

Animals were anesthetized with 50 mg/kg pentobarbital by an intraperitoneal injection before tracheotomy. BLM (1 mg/kg; Wako Pure Chemical Industries, Osaka, Japan) or LPS (2.5 μg/body or 50 μg/body; Chondrex, Redmond, WA) was dissolved in 100 μl of saline and then instilled intratracheally with a 27-gauge needle [49]. PARP inhibitors, PJ34 and 3-aminobezamide (3-AB) were dissolved in saline, and then administered intraperitoneally 1 h before injection of BLM. The administration volume for PARP inhibitors was 50 μl/10 g body weight.

2.3. Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed by cannulating the trachea with a 20-gauge needle and infusing the lungs 5 times with 1 ml of physiological buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) in order to measure inflammatory cell numbers and prepare AMs, or once with 500 μl of PBS to measure cytokines and total protein.

2.4. Measurement of inflammatory cell numbers

BAL fluid was centrifuged ($150 \times g$) for 10 min at 4 °C. The cells obtained were resuspended in 500 μl of PBS containing 0.1% BSA and used to measure the total cell count and cell fraction. In order to measure the cell fraction, cells were centrifuged onto a glass slide at $20 \times g$ for 4 min using a Floating cell collector (Tomy Company, Tokyo, Japan), and stained with May–Giemsa staining. At least 200 cells were examined for differential cell fractions.

2.5. Measurement of inflammatory cytokines and total protein in BAL fluid

The supernatants of BAL fluid after centrifugation ($150 \times g$) for 10 min were used as samples. The levels of cytokines including macrophage inflammatory protein-2 (MIP-2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), keratinocyte-derived cytokine (KC), and total protein were measured using the respective ELISA Kits (R&D Company, Minneapolis, MN) or DC Protein Assay Kits® (Bio-Rad laboratories, Hercules, CA) according to the manufacturer's instructions.

2.6. Preparation of AMs

BAL fluid was centrifuged ($150 \times g$) for 10 min at 4 °C. The cells collected were dispersed with RPMI-1640 medium containing 10% FBS, plated on glass-bottomed dishes (for Ca^{2+} measurements) or 48-well culture plates (for cytokine secretion measurements), and incubated at 37 °C for 1 h. The non-adherent cells were removed by washing with RPMI-1640 medium containing 10% FBS. The purity of AMs was confirmed by May–Giemsa staining (> 99%).

2.7. Preparation of primary alveolar epithelial cells and lung fibroblasts

Primary alveolar epithelial cells (AECs) and fibroblasts were isolated by enzymatic digestion using dispase and collagenase, as described previously [36]. Harvested cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) containing 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate for 3 days. The purities of AECs and lung fibroblasts were confirmed by an immunohistochemical analysis using a rabbit anti-E-cadherin antibody (BD Biosciences, Franklin Lakes, NJ) and mouse anti-α-SMA antibody (Sigma-Aldrich, St. Louis, MO) (> 95%), respectively.

2.8. Isolation of bone marrow PMNs

Mouse bone marrow PMNs were isolated from femurs by a density gradient with centrifugation using Percoll® (GE Healthcare, UK Ltd., England), as described previously [26]. The purity of PMNs was confirmed as Gr-1-positive cells by flow cytometry (> 91%) [26].

2.9. Reverse-transcription PCR (RT-PCR)

Total RNA was obtained using TRIzol® regent (Life Technologies, Carlsbad, CA). The expression of TRPM2 mRNA and glyceraldehyde-3-