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Full paper

Tyrphostin AG-related compounds attenuate H_2O_2-induced TRPM2-dependent and -independent cellular responses

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ABSTRACT

Purpose: TRPM2 is a Ca^{2+}-permeable channel that is activated by H_2O_2. TRPM2-mediated Ca^{2+} signaling has been implicated in the aggravation of inflammatory diseases. Therefore, the development of TRPM2 inhibitors to prevent the aggravation of these diseases is expected. We recently reported that some Tyrphostin AG-related compounds inhibited the H_2O_2-induced activation of TRPM2 by scavenging the intracellular hydroxyl radical. In the present study, we examined the effects of AG-related compounds on H_2O_2-induced cellular responses in human monocytic U937 cells, which functionally express TRPM2.
Methods: The effects of AG-related compounds on H_2O_2-induced changes in intracellular Ca^{2+} concentrations, extracellular signal-regulated kinase (ERK) activation, and CXCL8 secretion were assessed using U937 cells.
Results: Ca^{2+} influxes via TRPM2 in response to H_2O_2 were blocked by AG-related compounds. AG-related compounds also inhibited the H_2O_2-induced activation of ERK, and subsequent secretion of CXCL8 mediated by TRPM2-dependent and -independent mechanisms.
Conclusion: Our results show that AG-related compounds inhibit H_2O_2-induced CXCL8 secretion following ERK activation, which is mediated by TRPM2-dependent and -independent mechanisms in U937 cells. We previously reported that AG-related compounds blocked H_2O_2-induced TRPM2 activation by scavenging the hydroxyl radical. The inhibitory effects of AG-related compounds on TRPM2-independent responses may be due to scavenging of the hydroxyl radical.

1. Introduction

The second member of the transient receptor potential (TRP) melastatin subfamily, TRPM2, is a Ca^{2+}-permeable channel and is activated by oxidative stress induced by reactive oxygen species (ROS) such as H_2O_2.[1] It has been suggested that the activation of H_2O_2-induced TRPM2 is mediated by ADP-ribose production via poly(ADP-ribose) polymerase-1 (PARP-1) and poly(ADP-ribose) glycohydrolase (PARG) pathway in the nucleus.[2,3] We also recently demonstrated that H_2O_2-induced TRPM2 activation was mediated by the intracellular production of the hydroxyl radical via the reaction between H_2O_2 and intracellular Fe^{2+} (Fenton reaction).[4,5]

TRPM2 is expressed in neuronal cells, myocytes, pancreatic beta cells, and immunocytes such as T lymphocytes, monocytes/macrophages, and neutrophils.[6–14] The Ca^{2+} influxes via TRPM2 in response to ROS are suggested to induce not only cell death[1] but also various cellular responses such as cytokine production. Recent studies using *Trpm2* knockout (KO) mice have implicated TRPM2-dependent cellular responses in the aggravation of various diseases including inflammatory diseases and ischemia-reperfusion injury.[12,15–24] Thus, TRPM2 is a molecular entity that links ROS generated under pathological conditions and cellular responses, leading to the exacerbation of diseases.

We recently demonstrated that Tyrphostin AG490 attenuated H_2O_2-induced TRPM2 activation.[25] Although AG490 is known as a

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Janus kinase 2 (JAK2) inhibitor, its inhibitory effects on H_2O_2-induced TRPM2 activation have been attributed to scavenging of the intracellular hydroxyl radical, and not to the inhibition of JAK2 tyrosine kinase. Furthermore, we identified two other Tyrphostin AG-related compounds, AG555 and AG556, as stronger TRPM2 inhibitors than AG490.[26] However, these experiments were performed using human embryonic kidney (HEK) 293 cells stably expressing TRPM2 (TRPM2-expressing HEK cells), and it currently remains unclear whether these AG compounds attenuate the cellular responses attributed to the activation of TRPM2. We previously found that Ca^{2+} influxes via TRPM2 in response to ROS induced the production of the chemokine CXCL8 following the induction of CXCL8 mRNA via the extracellular signal-regulated kinase (ERK)/NF-κB pathway in human monocytic U937 cells.[12] U937 cells are a useful model for investigating the inhibitory effects of AG compounds on TRPM2-dependent cellular responses.

In the present study, we examined the effects of AG-related compounds on TRPM2-dependent cellular responses using human monocytic U937 cells, and found that AG-related compounds inhibited not only the TRPM2-dependent, but also TRPM2-independent cellular responses induced by H_2O_2.

2. Materials and methods

2.1. Cell culture

Human monocytic U937 cells (American Type Culture Collection) were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 37 °C under 5% CO_2. TRPM2-expressing HEK cells[26] were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin at 37 °C under 5% CO_2.

2.2. Measurement of changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$)

Changes in $[Ca^{2+}]_i$ were measured using the Ca^{2+} indicator, fura-2, as previously described.[5] Briefly, cells were transferred to coverslips precoated with poly-L-lysine (BD Biosciences), and were loaded with 5 μM fura-2/AM in culture medium containing 10% FBS at 37 °C for 40 min. Cells were then washed with HEPES-buffered saline (HBS) containing 107 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 11.5 mM glucose, and 20 mM HEPES, which was adjusted to pH 7.4 using NaOH. Coverslips were then placed in a perfusion chamber mounted on an inverted microscope equipped with a cool SNAP CCD camera (Photometrics). Fura-2 fluorescence images of cells were then recorded at 10-s intervals and analyzed using a video image analysis system (Mete Flour; Nippon Roper). Fluorescence at 510 nm (bandwidth: 50 nm) was observed after alternate excitation of fura-2 at 340 nm (bandwidth, 10 nm) and 380 nm (bandwidth, 13 nm).

2.3. Analysis of ERK activity

The activation of ERK was measured using Western blots with an anti-(phospho-ERK 1/2) antibody (Cell Signaling). Cells were lysed in lysis buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% NP-40) for 10 min on ice. After centrifugation at 20,000×*g* for 10 min, lysates were subjected to 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The total amount of ERK 1/2 was detected using an anti-ERK 1/2 polyclonal antibody (Cell Signaling). The intensity of each band was measured using ImageJ software.

2.4. Measurement of CXCL8 concentrations

Cell suspensions were centrifuged at 200×*g* at 4 °C for 5 min, and supernatants were collected. The secretion of the CXCL8 protein was quantitatively assayed by a Human CXCL8/IL-8 Quantikine ELISA Kit (R&D Systems) according to the manufacturer's instructions.

2.5. LDH leakage

Cell suspensions were centrifuged at 200×*g* at 4 °C for 5 min, and supernatants were collected in order to assess leaked lactate dehydrogenase (LDH) activity. The remaining cells were lysed in 1% Triton X-100, and centrifuged at 20,000×*g* for 10 min. Supernatants were then collected to evaluate the remaining intracellular LDH activity. The LDH activities of samples were measured using a LDH cytotoxicity detection kit (Takara) according to the manufacturer's instructions. Percent LDH leakage was calculated as leaked LDH activity/(leaked LDH activity + intracellular LDH activity).

2.6. Statistical analysis

All data are expressed as means ± S. E. M. Data were accumulated under each condition from 3 to 4 independent experiments. The significance of differences was evaluated using the *t*-test for comparisons between two mean values.

3. Results

3.1. AG490, AG555, and AG556 attenuate Ca^{2+} influxes via TRPM2 activated by H_2O_2 in human monocytic U937 cells

The activation of TRPM2 induced by H_2O_2 in TRPM2-expressing HEK 293 cells was blocked by AG490, AG555, and AG556.[26] Ca^{2+} influxes in response to H_2O_2 were previously found to be mediated by the activation of TRPM2 in U937 cells.[12] We initially investigated whether AG490, AG555, and AG556 inhibit TRPM2 activation in U937 cells. Similar to TRPM2-expressing HEK 293 cells, $[Ca^{2+}]_i$ increases in response to 100 μM H_2O_2 were completely blocked by 10 μM AG490, AG555, and AG556 in U937 cells (Fig. 1A). The concentration relationship of these compounds was examined. All these compounds concentration-dependently reduced H_2O_2-induced TRPM2 activation. The half maximal inhibitory concentrations (IC_{50}) of AG490, AG555, and AG556 were 0.35 μM, 0.16 μM, and 0.065 μM, respectively (Fig. 1B). We previously assessed the inhibitory effects of other Tyrphostin AG-related compounds, AG9, AG30, AG43, AG82, and AG538, on H_2O_2-induced TRPM2 activation in TRPM2-expressing HEK 293 cells.[26] Although AG9, AG30, and AG43 had no inhibitory effects on TRPM2 activation, AG82 and AG538 slightly reduced H_2O_2-induced TRPM2 activation in TRPM2-expressing HEK 293 cells. Similar to TRPM2-expressing HEK cells, $[Ca^{2+}]_i$ increases induced by H_2O_2 were not reduced by 10 μM AG9, AG30, or AG43 (Fig. 1C and D). The activation of TRPM2 induced by H_2O_2 was completely blocked by 10 μM AG82 and AG538 (Fig. 1C and D).

3.2. Inhibitory effects of AG490, AG555, and AG556 on H_2O_2-induced ERK activation

The activation of ERK in response to H_2O_2 was observed in the absence of extracellular Ca^{2+} (Fig. 2A). On the other hand, it was enhanced in the presence of extracellular Ca^{2+} (Fig. 2A). The enhanced activation of ERK was previously reported to be mediated by Ca^{2+} influxes via TRPM2.[12] We subsequently investigated the inhibitory effects of AG-related compounds on TRPM2-dependent