NF-κB inhibitor DHMEQ inhibits titanium dioxide nanoparticle-induced interleukin-1β production: Inhibition of the PM2.5-induced inflammation model

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Received May 25, 2018; Accepted September 6, 2018

DOI: 10.3892/mmr.2018.9533

Abstract. PM2.5 is a particle with a diameter <2.5 μm that is often involved in air pollution. Nanoparticles <100 nm are thought to invade the trachea and lungs to cause inflammation, possibly through the activation of macrophages. On the other hand, titanium dioxide (TiO_2) particles can be used in models of nano-micro-sized particles, as one can prepare the particles with such sizes. TiO_2 particles are classified into Rutile, Anatase, and Brookite types by their crystal structure. Among them, Anatase-type TiO_2 particles with a primary diameter of 50 nm (A50) were reported to induce interleukin (IL)-1β production and secretion effectively in phorbol 12-myristate 13-acetate-treated human monocytic leukemia THP-1 cells (THP-1 macrophages). We previously designed and synthesized dehydroxymethyl-epoxyqinomicin (DHMEQ) as an inhibitor of NF-κB. The present study investigated whether the NF-κB inhibitor DHMEQ inhibits TiO_2 nanoparticle-induced IL-1β production in THP-1 macrophages, and determined the mechanism. As a result, DHMEQ inhibited A50-induced IL-1β secretion in ELISA assays at nontoxic concentrations. It decreased the expression of IL-1β mRNA, which was dependent on NF-κB. Although NLR family pyrin domain containing 3 (NLRP3)-inflammasome-caspase-1 activation is required for the maturation of IL-1β, and DHMEQ reduced the NLRP3 mRNA expression and caspase-1 activity; a caspase-1 inhibitor did not influence the A50-induced IL-1β production. Therefore, it is likely that inhibition of pro-IL-1β expression by DHMEQ may be sufficient to inhibit mature IL-1β production. Thus, DHMEQ may be useful for the amelioration of inflammation in the trachea and lungs caused by inhalation of PM2.5.

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Key words: PM2.5, titanium dioxide, dehydroxymethyl-epoxyqinomicin, nuclear factor-κB, interleukin-1β, THP-1 macrophage

Introduction

PM2.5 is a particle of diameter less than 2.5 μm common in air pollution. It can be found in engine exhaust gas, and consists mainly of sulfur oxides (SOx) and nitrogen oxides (NOx). Because of its small size, the PM2.5 particle can penetrate into the trachea and lungs by inhalation, and it is considered to cause damage to the respiratory systems (1). Meanwhile particles of diameter less than 100 nm are called nanoparticles. Nanoparticles in PM2.5 were reported to effectively induce ROS production and inflammatory reactions in the body (2).

A model of PM2.5 nanoparticles can be prepared employing titanium dioxide (TiO_2). TiO_2 particles are classified into Rutile, Anatase, and Brookite types by their crystal structure. Rutile and Anatase types are often used in sunscreen, food additives and photo-catalytic agents. Moreover, when added to cultured human fibroblasts or lung cancer cells, Anatase-type nanoparticles induced cell death about 100 times more effectively than the Rutile type (3). The size of insoluble nanoparticles should be important for each biological effect (4). Recently, Tada-Oikawa *et al* (5) reported that the Anatase type TiO_2 nanoparticle of 50 nm diameter called A50 effectively induced IL-1β production in differentiated human monocytic leukemia cells. A50 showed stronger activity than other tested TiO_2 nanoparticles with a different crystal type or different size of particles.

We previously designed and synthesized dehydroxymethyl-epoxyqinomicin (DHMEQ) based on the structure of antibiotic epoxyqinomicin C as an inhibitor of NF-κB (6,7). It binds to the specific cysteine residues of NF-κB components, including p65, RelB, cRel, and p50, to inhibit their DNA binding (8,9). It causes irreversible inhibition of NF-κB when added to the cultured cells (10), since it binds to the cysteine residue covalently (11). It was shown to ameliorate various animal

models of inflammation and cancer without showing any side effect (12,13). Previously, it was shown to inhibit lipopolysaccharide (LPS)-induced inflammatory cytokine secretions in mouse monocyte leukemia RAW264.7 cells (14) and mouse primary culture macrophages (15). Recently, we reported that DHMEQ inhibited IL-6 and MCP-1 expressions in primary culture human peritoneal mesothelioma cells, either with or without stimulation (16).

In the present study we looked for the signaling inhibitors of low molecular weight that inhibit TiO_2 nanoparticle-induced production of IL-1β. As a result, we found that DHMEQ inhibited the A50-induced IL-1β production in differentiated human monocytic leukemia THP-1 cells without toxicity, possibly by inhibition of transcription alone.

Materials and methods

Materials. The TiO_2 particles were purchased from mkNano, Mississauga, ON, Canada. A50 (anatase type TiO_2 with a primary diameter of 50 nm) was prepared as reported previously (5). DHMEQ was synthesized in our laboratory as previously described (17). Cytochalasin D is an inhibitor of phagocytosis, and it was purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). We used KCl (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to increase the extracellular potassium ion concentration. We purchased a caspase-1 inhibitor, zYVAD-FMK, from Abcam (Cambridge, UK). N-acetylcysteine was used for anti-oxidant which was obtained from Wako Pure Chemical Industries, Ltd.

Cell culture and viability measurement. Human monocytic leukemia THP-1 cells (RIKEN BioResource Center, Tsukuba, Japan) were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum and penicillin/streptomycin at 37˚C in a humidified incubator with 5% CO_2. THP-1 cells were seeded at $1.5x10^4$ cells/well onto 96-well plates and differentiated into macrophage-like cells using PMA. The cells were treated with 0.1 μg/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich; Merck KGaA) for 72 h. Cell viability was evaluated by trypan blue (Sigma-Aldrich; Merck KGaA) exclusion assay.

Measurement of IL-1β production. THP-1 macrophages were exposed to different concentrations of the suspended A50 particles for 24 h at 37˚C. The cell culture supernatant was collected and stored at -20˚C until cytokine analysis. IL-1β concentration in the medium was measured by ELISA kit (BioLegend, Inc., San Diego, CA, USA) as described previously (5). The absorbance was measured at 450 nm by the micro plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

RNA isolation and semi-quantitative (q)-PCR. Total RNA from culture cells was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Reverse transcription was carried out at 37˚C for 120 min with High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The prepared cDNA was used for PCR amplification with Taq DNA polymerase (Toyobo Life Science, Osaka, Japan). The number of PCR cycles for each product was determined after the confirmation of amplification efficacy. After the determination of PCR cycles and annealing temperatures, sqPCR was carried out. The linear primers used in this study and experimental conditions are as follows: IL-1β, 5'-TGAGCTCGCCAGTGAAATGA-3' (forward) and 5'-AACACGCAGGACAGGTACAG-3' (reverse), 24 cycles, 55˚C; NLRP3, 5'-GGAGGAGGACTTCGTGCAAA-3' (forward) and 5'-CCCGGCAAAAACTGGAAGTG-3' (reverse), 26 cycles, 55˚C, and β-actin, 5'-CTTCTACAATGAGCTGCGTG-3' (forward) and 5'-TCATGAGGTAGTCAGTCAGG-3' (reverse), 21 cycles, 58˚C. The PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized with a UV illuminator.

Measurement of NF-κB activity. THP-1 cells were seeded at $3.0x10^6$ cells/dish onto 60 mm dishes and differentiated to macrophages using PMA as described above. THP-1 macrophage cells were treated with DHMEQ for 2 h then added A50 for 4 h. The nuclear extracts were prepared as described before (18) using Nuclear Extract kit (Active Motif Japan, Tokyo, Japan). The cells were washed with ice-cold PBS containing phosphatase inhibitors and added to the same solution. The cells were removed from the dish by scraping and transferred to a conical tube, then centrifuged for 10 min at 200 x g. The cell pellets were used as the total cell extracts. They were resuspended in 500 μl of 1X Hypotonic Buffer and incubated on ice for 15 min. After the incubation, they were centrifuged for 30 sec at 14,000 x g. The pellets were used for the preparation of nuclear fraction. They were resuspended in 30 μl of 1X Complete Lysis Buffer and incubated on ice for 30 min on a rocking platform shaker at 150 rpm. After the incubation on ice the lysate was centrifuged for 10 min at 14,000 x g. The resulting supernatant was used for the nuclear extract.

Then, the DNA binding activity of NF-κB in nuclear extracts was measured with the TransAM NF-κB p65 Transcription Factor Assay kit (Active Motif Japan) according to the manufacturer's instructions.

Measurement of caspase-1 activity. THP-1 cells were seeded at $1.5x10^6$ cells/dish onto 60 mm dishes and differentiated to macrophages using PMA as described above. THP-1 macrophages were treated with DHMEQ for 2 h, and then A50 was added. The cells were cultured for 24 h. They were collected by centrifugation at 250 x g for 10 min after the detachment. The supernatant was removed and discarded, while the cell pellet was lysed by the addition of 50 μl Lysis Buffer. The cell lysate was incubated on ice for 10 min and centrifuged at 10,000 x g for 1 min. The supernatant was used for the measurement of caspase-1 activity. To evaluate the caspase-1 activity, Caspase-1/ICE Colorimetric Assay Kit (R&D Systems, Inc., Minneapolis, MN, USA) was used, and it was used as described in the manufacturer's instructions.

Statistical analysis. All results were presented as the mean ± standard deviation. The significance of differences among groups was analyzed by one-way analysis of variance followed by Dunnett's post hoc test using Excel software v.2013 (Microsoft Corporation, Redmond, WA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

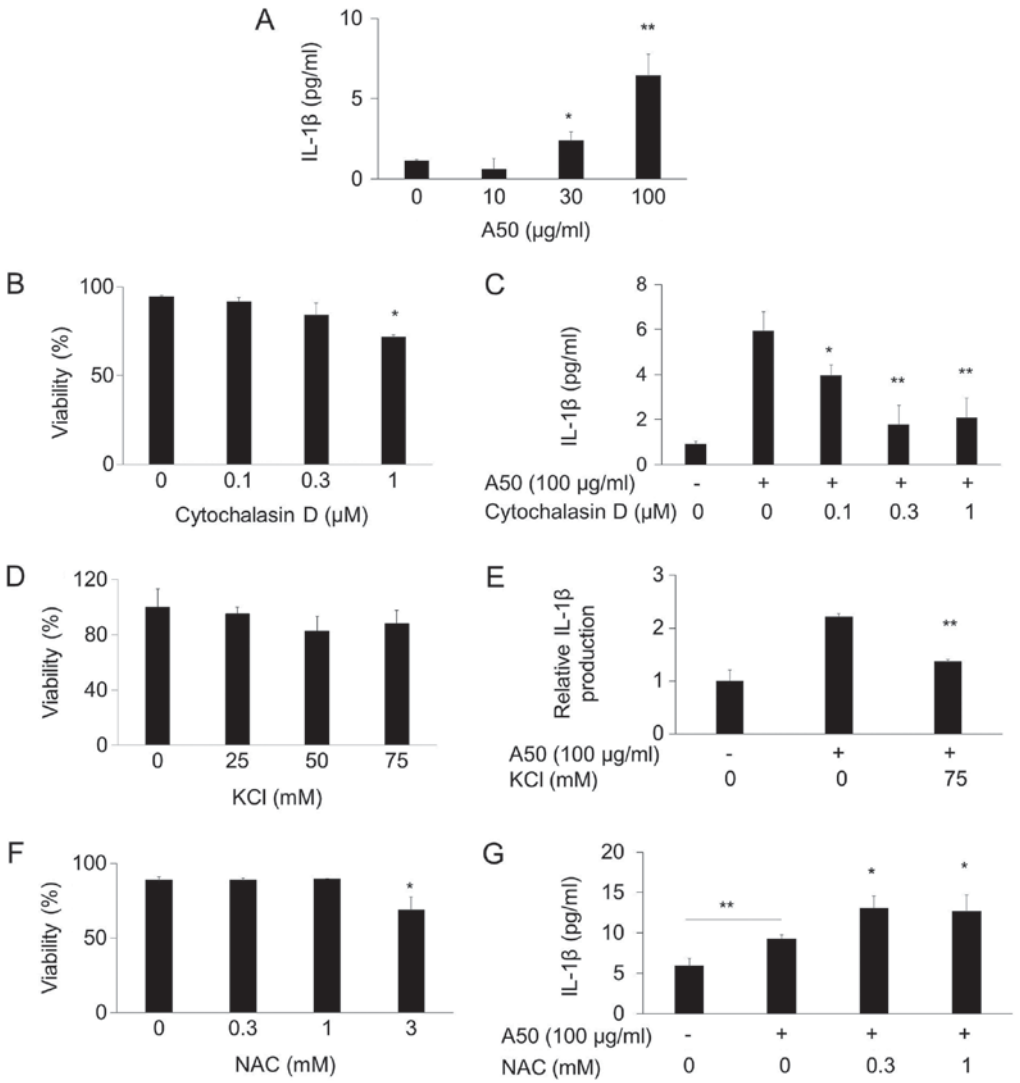

Figure 1. Inhibition of A50-induced IL-1β production by cytochalasin D and extracellular KCl. (A) Induction of IL-1β production by A50 in differentiated human monocytic leukemia THP-1 cells (THP-1 macrophages). *P<0.05 and **P<0.01 vs. control (0 μg/ml). (B) Effect of cytochalasin D on the viability of THP-1 macrophages. Cytochalasin D was added to the cells for 24 h. *P<0.05 vs. control. (C) Inhibition of IL-1β production by cytochalasin D. The cells were incubated for 24 h. *P<0.05 and **P<0.01 vs. A50 control (+100 μg/ml A50). (D) Effect of extracellular K^+ ions on the viability of THP-1 macrophages. KCl was added to the cells for 24 h. (E) Inhibition of IL-1β production via the addition of KCl. The cells were incubated for 24 h. **P<0.01 vs. A50 control (+100 μg/ml A50). (F) Effect of NAC on cell viability. NAC was added to the cells for 24 h. *P<0.05 vs. control. (G) Effect of NAC on A50-induced IL-1β production. The cells were incubated for 24 h. *P<0.05 and **P<0.01 vs. A50 bar. IL-, interleukin-; NAC, N-acetylcysteine.

Results

Inhibition of A50-induced IL-1β production by cytochalasin D and extracellular potassium ion. We evaluated the IL-1β secretion by measuring the amount in medium using ELISA. As reported before (5), A50 induced IL-1β production in PMA-treated THP-1 cells (Fig. 1A). Cytochalasin D was not toxic at 1 μM (Fig. 1B), and inhibited the IL-1β production at 0.1-1 μM (Fig. 1C). Thus, it is likely that A50 is incorporated into the cells by phagocytosis. K^+ efflux was reported to be necessary for IL-1β maturation (19). We found that extracellular potassium ion inhibited the increase of IL-1β secretion by A50 (Fig. 1D and E). Thus, it is likely that K^+ efflux is essential for the A50-induced IL-1β secretion. On the other hand, N-acetylcysteine did not lower and rather increased the A50-induced IL-1β production at the nontoxic concentrations (Fig. 1F and G).

Inhibition of A50-induced IL-1β production by DHMEQ. DHMEQ (Fig. 2A) showed no toxicity below 10 μg/ml (Fig. 2B). It was shown to inhibit A50-induced IL-1β secretion at 3-10 μg/ml (Fig. 2C). Then, we studied the effect of IL-1β mRNA expression. A50 did not enhance the mRNA expression, but DHMEQ clearly inhibited the expression (Fig. 2D). Next, we confirmed the inhibition of NF-κB. As shown in Fig. 2E, A50 did not significantly increase the cellular NF-κB activity. But DHMEQ clearly lowered the NF-κB activity in 2 h (Fig. 2E).

Effect of caspase-1 inhibitor on A50-induced IL-1β secretion. IL-1β maturation is often activated by inflammasome/caspase-1 mechanism. We found that cellular caspase-1 activity was significantly increased by A50, which was inhibited by DHMEQ (Fig. 3A). NLRP3 mRNA expression was not increased by A50, but inhibited by DHMEQ (data not shown).

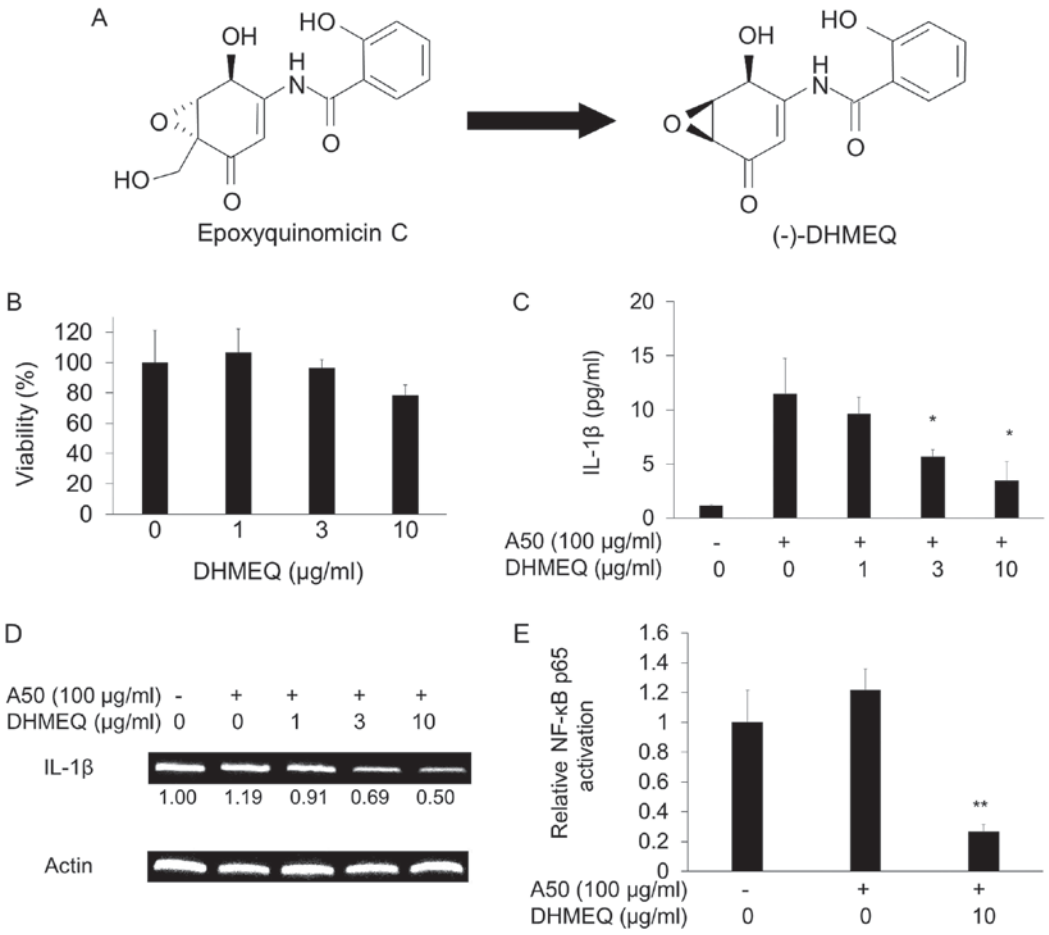

Figure 2. Inhibition of IL-1β secretion, expression, and NF-κB by DHMEQ. (A) Structure of DHMEQ derived from epoxyquinomicin C. (B) Effect of DHMEQ on the viability of THP-1 macrophages. DHMEQ was added to the cells for 24 h. (C) Inhibition of IL-1β secretion by DHMEQ. The cells were incubated for 24 h. (D) Inhibition of IL-1β mRNA expression by DHMEQ. The cells were incubated for 4 h, and total RNA was prepared for polymerase chain reaction analysis. (E) Inhibition of cellular NF-κB by DHMEQ. The cells were incubated for 4 h, and the nuclear extract was prepared. *P<0.05 and **P<0.01 vs. A50 control (+100 μg/ml A50). IL-, interleukin-; NF-, nuclear factor; DHMEQ, dehydroxymethyl-epoxyqinomicin.

To study the functional involvement of caspase-1 decrease, we employed zYVAD-FMK. It did not lower the viability even at 10 μM (Fig. 3B). Unexpectedly, the inhibitor did not influence the A50-induced IL-1β secretion at 1-10 μM (Fig. 3C). When added with DHMEQ, it did not show any influence again (Fig. 3D). Thus, it is unlikely that caspase-1 decrease is involved in the mechanism for inhibition of IL-1β secretion by DHMEQ. Therefore, inhibition of pro-IL-1β mRNA transcription by DHMEQ would be sufficient to inhibit A50-induced K^+ efflux-dependent IL-1β secretion (Fig. 4).

Discussion

The TiO_2 model may be too much simplified as a model of PM2.5. But so far, it is considered to be a suitable model, since TiO_2 nanoparticles have been shown to induce lung inflammation in mice (20) and IL-1β formation in macrophages (5).

We found that A50-induced IL-1β secretion was inhibited by a phagocytosis inhibitor, cytochalasin D. K^+ efflux was also found to be essential for the efficacy of A50. But clinical use of cytochalasin D and KCl are known to be difficult. On the other hand, DHMEQ has been widely used for the suppression of various inflammation and neoplastic disease models in animals. Therefore, it may be useful for the treatment of PM2.5-induced inflammation in the trachea and lungs.

We employed TiO_2 nanoparticle of 50 nm called A50, which is slightly smaller than general microorganisms. Therefore, it is likely to be incorporated into the mammalian cells by phagocytosis to stimulate cellular activity. In fact, the efficacy of A50 was inhibited by cytochalasin D, which itself inhibits phagocytosis (Fig. 1C).

A50 did not increase the NF-κB activity (Fig. 2D). This may be because the THP-1 cells are treated with PMA for 3 days to obtain THP-1 macrophages. PMA is known to activate NF-κB (21). However, DHMEQ clearly inhibited the basal NF-κB activity (Fig. 2D). Thus, the basal NF-κB activity in THP-1 macrophages should be necessary for the A50 activity on cytokine production. DHMEQ should inhibit cellular NF-κB in the nonspecific manner on upstream signaling, since it was shown to inhibit the cellular NF-κB in unstimulated adult T-cell leukemia cells (22) and chronic lymphocytic leukemia cells (23). The nonspecific inhibition is due to the mechanism that DHMEQ inhibits the final process of NF-κB activation, which is NF-κB-DNA binding (8,9). The PMA-treated THP-1 cells possess limitation for the mechanistic study with DHMEQ, since NF-κB activity is already activated by PMA. Then, it is important to use PMA-untreated

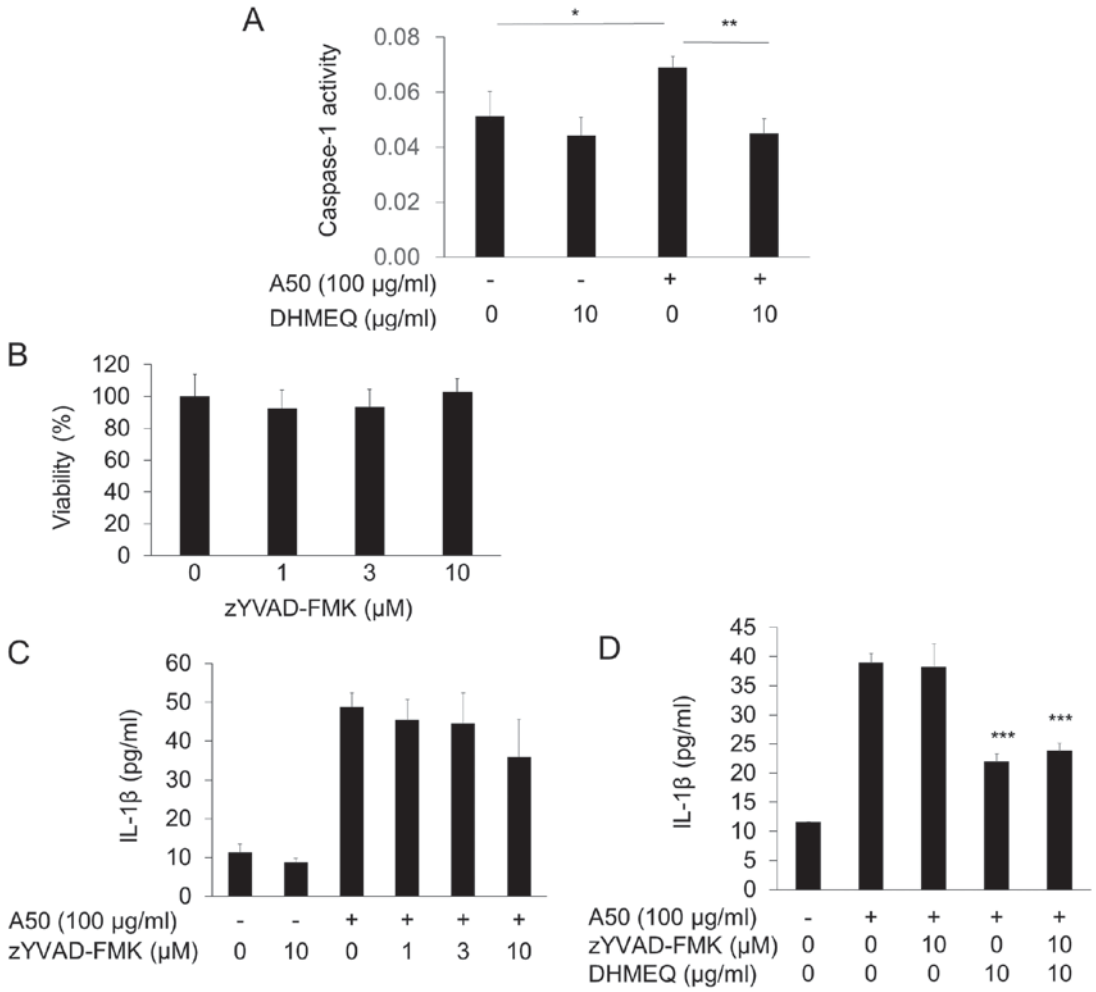

Figure 3. Effect of the caspase-1 inhibitor zYVAD-FMK on IL-1β production. (A) Inhibition of A50-induced caspase-1 activation by DHMEQ. The cells were incubated for 24 h. *P<0.05 and **P<0.01, as indicated. (B) Effect of zYVAD-FMK on cell viability. The cells were incubated for 24 h. (C) Effect of zYVAD-FMK on A50-induced IL-1β production in THP-1 macrophages. The cells were incubated for 24 h. (D) Effect of zYVAD-FMK on A50-induced IL-1β production when combined with DHMEQ. ***P<0.001 vs. A50 control (+100 μg/ml A50). IL-, interleukin-; DHMEQ, dehydroxymethyl-epoxyqinomicin.

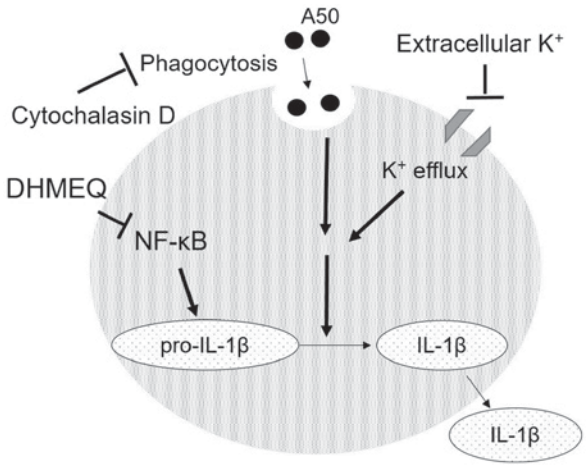

Figure 4. Possible mechanism underlying the inhibition of A50-induced IL-1β production by DHMEQ. Inhibition of pro-IL-1β mRNA expression is likely to be sufficient to inhibit the production/secretion. IL-, interleukin-; NF-, nuclear factor; DHMEQ, dehydroxymethyl-epoxyqinomicin

cells. Since intact THP-1 cells were reported to be nonresponsive to Anatase-type TiO_2 nanoparticles (24), we have tried to use mouse monocytic leukemia RAW264.7 cells that are LPS-sensitive without differentiation. However, they were not responsive to A50 on IL-1β production. The mechanism of A50 downstream signaling has not been fully elucidated. In future, we should screen A50-sensitive macrophage-like cells from other sources including primary culture of macrophages to study the downstream signaling.

We measured secretion of IL-1β by ELISA. The secretion should depend on the mRNA expression and post-translational maturation. The former is known to be dependent on NF-κB. In fact, it was inhibited by DHMEQ, as shown in Fig. 2C. On the other hand, inflammasome/caspase-1 mechanisms is known to be necessary for the maturation of IL-1β from its precursor protein (25). This system is a main signaling pathway in pyroptosis, a programmed cell death (26). One of the main components of inflammasome is NLRP3. We found that although A50 did not increase NLRP3 expression, DHMEQ lowered its mRNA expression (data not shown). Moreover, expression of a downstream component caspase-1 was activated by A50 and inhibited by DHMEQ (Fig. 3A). Inflammasome/caspase-1 axis to activate IL-1β production is known to be dependent on K^+ efflux (19). In fact, we found that extracellular K^+ ion inhibited the A50-induced IL-1β production

(Fig. 1E). Therefore, we studied the functional involvement of caspase-1 using its chemical inhibitor zYVAD-FMK. We used this inhibitor at 1-10 mM as shown in Fig. 3B, because it was reported to be used at below 10 mM (24). However, zYVAD-FMK did not inhibit A50-induced IL-1β production with or without DHMEQ. Therefore, it is unlikely that inhibition of caspase-1 is involved in the mechanism of inhibition by DHMEQ in our experimental system.

N-acetylcysteine did not inhibit A50-induced IL-1β production in our study (Fig. 1G), although Tada-Oikawa *et al* (5) reported that A50 induced ROS production in THP-1 macrophages. Moreover, Yazdi *et al* (27) demonstrated that the chemical ROS scavenger diminished IL-1β production triggered by TiO_2 nanoparticles in THP-1 macrophages, suggesting that IL-1β production is caused by ROS in TiO_2 nanoparticle-treated cells. On the other hand, other reports showed that ROS was not essential for IL-1β production via the Nlrp3 inflammasome (28,29). ROS might not be the main contributing factor of particle-induced IL-1β production in THP-1 macrophages because the ROS level was increased to some extent after the exposure to all TiO_2 particles (5). Our results indicated that ROS may not be functionally involved in the A50-induced IL-1β production. In total, it is still controversial whether ROS is involved in the mechanism of TiO_2 nanoparticle-induced IL-1β production.

DHMEQ was shown to be nontoxic in animals in many *in vivo* experiments. DHMEQ ointment inhibits the atopic dermatitis model in mice (30,31), and is now being developed for the treatment of skin inflammation. Meanwhile it inhibited the LPS-induced sepsis model in animal experiments by suppression of tumor necrosis factor (TNF)-α production (32). DHMEQ also inhibited allergic inflammation and airway remodeling in murine models of asthma (33). Therefore, it may be possible that DHMEQ can protect the patient from PM2.5-caused inflammation in the trachea and lungs.

Acknowledgements

Not applicable.

Funding

The present study was financially supported in part by JSPS Kakenhi (grant no. 17K01967), the AMED grant (grant no. JP18fk0310118)), and the Aichi Medical University Research Unit Fund.

Availability of data and materials

All data generated during the present study are included in this published article.

Authors' contributions

HF performed the majority of experiments. KU, HF and TU designed the experiments. KI and AI prepared the A50 (TiO_2) suspension. NK, STO, SI, NM, YN and TK contributed to the design of the methodology. HF and KU analyzed the data and wrote the paper.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The Department of Molecular Target Medicine is a fund-donated laboratory. It is supported financially by Shenzhen Wanhe Pharmaceutical Company (Shenzhen, China), Meiji Seika Kaisha, Ltd (Tokyo, Japan), Fukuyu Hospital (Nisshin, Japan), and Brunese Co., Ltd (Nagoya, Japan).

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