Afzelin suppresses proinflammatory responses in particulate matter-exposed human keratinocytes

JU HEE KIM\textsuperscript{1}, MINJEONG KIM\textsuperscript{1}, JAE MIN KIM\textsuperscript{1}, MI-KYUNG LEE\textsuperscript{2}, SEONG JUN SEO\textsuperscript{1} and KUI YOUNG PARK\textsuperscript{1}

Departments of \textsuperscript{1}Dermatology and \textsuperscript{2}Laboratory Medicine, Chung-Ang University Hospital, Seoul 06973, Republic of Korea

Received September 28, 2018; Accepted April 2, 2019

DOI: 10.3892/ijmm.2019.4162

Abstract. Particulate matter (PM), a widespread airborne contaminant, is a complex mixture of solid and liquid particles suspended in the air. Recent studies have demonstrated that PM induces oxidative stress and inflammatory reactions, and may cause certain skin diseases. Afzelin is a flavonoid isolated from \textit{Thesium chinense} Turcz, which has anti-inflammatory, anticancer and antibacterial properties. Therefore, the present study aimed to investigate if afzelin affected inflammatory responses in human keratinocytes exposed to PM. HaCaT cells were treated with PM (25 \( \mu \text{g/cm}^2 \)) in the presence or absence of afzelin (200 \( \mu \text{M} \)). Here, standard reference material 1649b was used as PM. Cell viability was assessed using the water-soluble tetrazolium salt-1 assay. The generation of reactive oxygen species (ROS) was measured using the dichloro-dihydro-fluorescein diacetate assay. Gene and protein expression were investigated using reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. Levels of secreted inflammatory cytokines were measured using ELISA. The results suggested that afzelin inhibited PM-induced proinflammatory cytokine mRNA expression and protein secretion in HaCaT cells. In addition, afzelin suppressed PM-induced intracellular ROS generation, and p38 mitogen-activated protein kinase and transcription factor activator protein-1 component c-Fos and c-Jun activation. The results indicated that afzelin exerts anti-inflammatory and antioxidant effects in PM-exposed HaCaT. Afzelin may have potential for preventing PM-induced inflammatory skin diseases.

Introduction

Particulate matter (PM) or particle pollutants are a complex mixture of solid and liquid particles of various sizes and composition and are a widespread airborne contaminant (1). Prevalence of PM increases with urbanization and industrialization (1). PM components that are harmful to human health include polycyclic aromatic hydrocarbons (PAHs), organic compounds, bacteria, metals and carbon particles (2-4). Numerous studies have demonstrated that PM induces oxidative stress and inflammatory reactions, and that PM exposure may cause various diseases following penetration and accumulation in respiratory and cardiovascular systems (5,6).

Skin serves as an important interface between the body and the environment, with the epidermal barrier preventing environmental stress and dehydration (3,7,8). Previously, several studies have demonstrated that PM causes skin barrier dysfunction and skin inflammatory diseases, including atopic dermatitis and psoriasis (3,9-13). PM promotes the generation of reactive oxygen species (ROS) and inflammation (2,13,14). Increased ROS levels serve an important role in DNA damage, whilst elevated oxidative stress may activate mitogen-activated protein kinases (MAPKs) and the transcription factor activator protein-1 (AP-1), which lead to the release of proinflammatory cytokines (7,15,16).

Afzelin (3-O-\( \alpha \)-L-rhamnopyranoside; Fig. 1A) is a flavonoid isolated from \textit{Thesium chinense} Turcz, which is widely distributed throughout Korea and China (17,18). Previous studies have suggested that afzelin has anti-inflammatory, anticancer and antibacterial properties (19-21), and possesses DNA-protective, antioxidant and anti-inflammatory properties in UVB-irradiated human skin cells (22,23).

However, effects of afzelin on PM-mediated responses have not yet been elucidated. Therefore, the present study aimed to investigate protective effects of afzelin in PM-exposed cells. It was reported that afzelin reduced ROS generation or attenuated proinflammatory responses.

Materials and methods

Materials. Standard reference material (SRM) 1649b, mainly composed of PAHs and dioxin (9), was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA) and dispersed in distilled water (240 \( \mu \text{g/ml} \)). Specific antibodies for western blot analysis, including anti-phosphorylated (p)-p38 MAPK (cat. no. 9211), anti-p38 MAPK (cat. no. 8690), anti-p-c-Fos (cat. no. 5248), anti-c-Fos (cat. no. 4384), anti-p-c-Jun (Ser63; cat. no. 2361), anti-p-c-Jun (Ser73; cat. no. 3270) and anti-c-Jun (cat. no. 9165).

Key words: afzelin, inflammation, keratinocytes, particulate matter, proinflammatory cytokines
were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-GAPDH (cat. no. sc-47724) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Secondary antibodies, goat anti-rabbit (cat. no. 31460) and goat anti-mouse IgG-horseradish peroxidase (cat. no. 31430) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Human interleukin (IL)-1α (cat. no. DY200-05), IL-1β (cat. no. DY201-05) and IL-6 (cat. no. DY206-05) ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Afzelin (purity, ≥98%) was purchased from Wuhan ChemFaces Biochemical Co., Ltd. (Wuhan, China).

Cell culture. HaCaT cells (American Tissue Cell Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin at 37˚C in a humidified 5% CO₂ atmosphere.

DNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from cells that were pretreated with afzelin (200 µM) for 24 h followed by PM (25 µg/cm²) for 2 h using TRIzol reagent (Wegene, Inc., Gyeongsan, South Korea) according to the manufacturer's protocol. cDNA was synthesized by RT using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) for 1 h at 42˚C. cDNA was amplified by qPCR with specific primers for IL-1α, IL-1β, IL-6 and GAPDH and assays were performed using PowerUp SYBR-Green Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermocycling conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, 60˚C for 30 sec and 72˚C for 30 sec. Results were normalized to GAPDH and quantified using the 2^ΔΔCt method (25). Primer sequences were as follows: IL-1α forward, 5'-ATCAGTACCTACGGCTGCT-3'; and reverse, 5'-TGGGTATCTCAAGGTATCC-3'; IL-1β forward, 5'-GGCCTCAAGGAAAGACCT-3' and reverse, 5'-TTC TGTTAGAGGAGCTGA-3'; IL-6 forward, 5'-GCCCTTC GGTCCATTGGCCT-3' and reverse, 5'-GCGAAGATGAG GATTGTC-3'; and GAPDH forward, 5'-GAGTCAACCGGAT TTGGTCT-3' and reverse, 5'-TTGATTTTGGAGGATCT CG-3'.

Western blot analysis. Cells (1.3x10⁴/well) were cultured in 6-well plates. At confluence, cells were pretreated with afzelin (200 µM) for 24 h and then incubated with PM (25 µg/cm²) for 1 h at 37˚C. Cells were lysed using 1% Triton-X radioimmunoprecipitation assay buffer (50 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris-HCl pH 8.0) containing a protease inhibitor cocktail (Complete™, EDTA-free protease inhibitor; Roche Diagnostics, Indianapolis, IN, USA). Cell debris was removed by centrifugation (10,000 x g, 10 min, 4˚C) and the protein concentration was determined using the bicinchoninic acid assay method. Equal amounts of protein (30 µg) were separated on 8% SDS-PAGE gels and proteins were transferred to nitrocellulose membranes and blocked with 5% skimmed milk for 1 h at 25˚C. Subsequently, membranes were incubated with primary antibodies (1:1,000) overnight at 4˚C, followed by horseradish peroxidase-conjugated secondary antibodies (1:2,000) for 1 h at 25˚C. Protein expression was detected using the EzWestLumi plus system (ATTO Corporation, Tokyo, Japan). Protein expression levels were quantified using Image J v1.8.0 (National Institutes of Health, Bethesda, MD, USA) and normalized to GAPDH.

ELISA. Proinflammatory cytokine levels were analyzed using ELISA. Cells (1.3x10⁴/well) were cultured in 6-well plates and were pretreated with afzelin (200 µM) for 24 h followed by incubation with PM (25 µg/cm²) for 24 h. Subsequently, conditioned media from the cells were collected and stored at -80˚C. Cytokines released by the cells were quantified using ELISA kits for human IL-1α, IL-1β and IL-6 according to the manufacturer's protocol.

Statistical analysis. In vitro assays were performed ≥3 times. Data are presented as the mean ± standard deviation. Data were analyzed using one-way analysis of variance with Bonferroni
post hoc tests. Statistical analysis was performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of PM and afzelin on HaCaT viability. To determine the cytotoxicity of PM and afzelin in HaCaT cells, cells were treated with various concentrations of PM (0, 10, 25, 50 or 100 µg/cm²) and afzelin (0, 50, 100 or 200 µM) for 24, 48 or 72 h measured using the water-soluble tetrazolium salt-1 assay. The ratio of surviving cells is relative to the untreated control. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01 vs. untreated control; ##P<0.01 vs. PM-treated. PM, particulate matter.

Afzelin exerts intracellular ROS scavenging effects on PM-treated HaCaT cells. To confirm effects of PM on intracellular ROS generation in HaCaT cells, cells were treated with PM (25 µg/cm²) for 2 h. Intracellular ROS levels were detected by measuring the fluorescence intensity of the oxidant-sensitive probe DCFH-DA over 120 min (Fig. 2A). PM treatment significantly increased intracellular ROS levels in HaCaT cells in a time-dependent manner compared with the untreated control. To examine the intracellular ROS scavenging effect of afzelin, cells were treated with afzelin (200 µM) for 2 h prior to or following exposure to PM (25 µg/cm²). As presented in Fig. 2B, afzelin significantly reversed PM-induced ROS generation suggesting that afzelin treatment may prevent and inhibit PM-induced intracellular ROS generation in HaCaT cells.

Afzelin affects AP-1 and p38 protein expression in PM-treated HaCaT cells. Western blotting was used to assess effects of afzelin on expression levels of the signaling molecules AP-1 and p38 MAPK. Cells were treated with PM (25 µg/cm²) for 1 h following pretreatment with afzelin (200 µM) for 24 h. Afzelin pretreatment significantly inhibited PM-induced phosphorylation of p38 MAPK and AP-1 components c-Fos and c-Jun (serine 63 and 73; Fig. 3).

Afzelin inhibits proinflammatory cytokine expression in PM-treated HaCaT cells. To investigate if afzelin inhibited

Figure 1. Effects of PM and afzelin on HaCaT viability. (A) Chemical structure of afzelin. Viability of HaCaT cells treated with (B) PM (0, 10, 25, 50 or 100 µg/cm²) or (C) afzelin (0, 50, 100 or 200 µM) for 24, 48 or 72 h measured using the water-soluble tetrazolium salt-1 assay. The ratio of surviving cells is relative to the untreated control. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01 vs. untreated control. PM, particulate matter.

Figure 2. ROS-scavenging effect of afzelin on HaCaT cells following PM treatment. (A) Relative intracellular ROS fluorescence intensity in HaCaT cells the presence of PM (25 µg/cm²). Measurements were performed every 15 min for 2 h. Fluorescence intensity was calculated relative to the control at 5 min. (B) Relative intracellular ROS fluorescence intensity at 2 h following treatment; cells were treated with afzelin (200 µM) or PM (25 µg/cm²) for 2 h or incubated with PM (25 µg/cm²) for 10 min followed by afzelin (200 µM) for 2 h and intracellular ROS levels were determined. ROS generation was calculated relative to the untreated control. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01 vs. untreated control; ##P<0.01 vs. PM-treated. ROS, reactive oxygen species; PM, particulate matter.
PM-induced proinflammatory cytokine expression, HaCaT cells were pretreated with afzelin (200 µM) for 24 h followed by PM (25 µg/cm²) for 2 or 24 h to determine mRNA or protein levels, respectively. RT-qPCR and ELISA analysis were performed to determine mRNA and protein levels, respectively, of IL-1α, IL-1β and IL-6. PM treatment significantly increased mRNA and protein levels of IL-1α, IL-1β and IL-6 in HaCaT cells compared with the untreated cells (Fig. 4). As presented in Fig. 4A, afzelin pretreatment significantly attenuated mRNA level increases of IL-1α compared with the PM-treated cells, while no significant changes were observed for IL-1β or IL-6. Furthermore, ELISA revealed that afzelin pretreatment significantly suppressed the PM-induced increase in IL-1α, but not IL-1β or IL-6 (Fig. 4B).

Figure 3. Effects of afzelin on transcription factor activator protein-1 components and p38 MAPK phosphorylation in HaCaT cells following PM treatment. Protein levels of p38 MAPK, c-Jun (serine 63 and 73) and c-Fos were analyzed by western blotting. (A) Western blot images for total and phosphorylated proteins of cells treated with PM (25 µg/cm²) for 1 h following afzelin (200 µM) pretreatment for 24 h and (B) quantification of protein levels. Data are presented as the mean ± standard deviation (n=3). **P<0.01 vs. untreated control; #P<0.05 vs. PM-treated. PM, particulate matter; MAPK, mitogen-activated protein kinase; p, phosphorylated; T, total.
Discussion

PM exposure has various adverse effects on human skin; the association between PM exposure and skin was elucidated by epidemiological studies connecting PM with skin inflammation, skin cancer and skin diseases, including atopic dermatitis, eczema and psoriasis (1,2,10,26). The current study used SRM 1649b as a standard PM, which is mainly composed of PAHs. PAHs possess the ability to damage the plasma membrane, alter cell physiology and induce cell death (9,27).

Afzelin is a flavonoid compound isolated from the Thesium chinense Turcz, which has been used in traditional Korean and Chinese medicine to treat inflammatory diseases (17,18) and has been reported to have anti-inflammatory, anticancer and antibacterial properties (19‑21). Recent reports demonstrated the anti‑inflammatory effects of afzelin in UVB‑irradiated skin cells (22,23). However, the effects of afzelin on PM exposure have not yet been clarified. Therefore, the present study investigated whether afzelin affected inflammatory responses of HaCaT cells exposed to PM.

Afzelin had no significant cytotoxicity at ≤200 µM and cells in subsequent experiments were treated with 200 µM afzelin. Various environmental pollutants can directly or indirectly produce ROS, and increased ROS levels serve a role in the pathogenesis of human skin disorders and reduce skin function (2,7). It was revealed that intracellular ROS generation was elevated in HaCaT cells treated with 25 µg/cm² PM. PM enhances oxidative stress by contributing to mitochondrial injury (28,29). Furthermore, increased oxidative stress activates the MAPKs and AP‑1 signaling pathways (7,15,16).

Findings of the current study suggested that afzelin significantly alleviated intracellular ROS generation in PM‑treated HaCaT cells. Many antioxidants exert activity by functioning as hydrogen or electron donors (30). It was suggested that the hydroxyl groups of afzelin exert a protective effect against oxidative stress, indicating that afzelin may protect skin keratinocytes from PM‑induced oxidative stress and regulate MAPKs and AP‑1 levels via oxidative stress management. It was investigated whether afzelin inhibited PM‑induced p38 MAPK and AP‑1 phosphorylation, which is associated with the regulation of proinflammatory cytokine release (7,15,16).

The current study demonstrated that afzelin pretreatment significantly inhibited the phosphorylation of p38 MAPK and AP‑1 components in PM‑treated HaCaT cells, indicating an anti‑inflammatory effect of afzelin that may be mediated by p38 MAPK and AP‑1 signaling. There are many processes between transcription and translation in PM‑induced inflammatory signaling and the protein and mRNA analysis time in the current study was determined by the highest expression of each target. Therefore, PM‑induced phosphorylation levels of p38 and AP‑1 were analyzed in HaCaT cells at different times. As several studies have reported that air pollutants enhance the secretion of proinflammatory cytokines (2,31‑33), the current study investigated whether afzelin suppressed the release of proinflammatory cytokines, including IL‑1α, IL‑1β and IL‑6, in PM‑treated HaCaT cells. Afzelin pretreatment attenuated mRNA and protein secretion levels of IL‑1α by keratinocytes causes skin diseases, including contact and atopic dermatitis (32,33). IL‑1β and IL‑6 levels were not significantly affected by afzelin pretreatment of PM‑treated HaCaT cells. Further studies are required to elucidate the anti-inflammatory activities of afzelin in animal models with PM‑induced atopic dermatitis.

In conclusion, the results of the present study indicated that afzelin exerts anti‑inflammatory effects in HaCaT cells exposed
to PM, potentially by inhibiting mRNA and protein expression of IL-1α. Anti-inflammatory properties of afzelin may be associated with downregulating the p38 MAPK and AP-1 signaling pathways. Afzelin demonstrated antioxidant activities by alleviating intracellular ROS generation in PM-treated HaCaT cells. The present findings suggested that afzelin may have the potential of preventing air pollution-induced inflammatory skin diseases.

Acknowledgements

Not applicable.

Funding

This research was supported by the Basic Science Research Foundation of Korea funded by the Ministry of Science, ICT & Future Planning (grant no. NRF-2017R1C1B1007523).

Availability of data and materials

The datasets analyzed in the study are available from the corresponding author on reasonable request.

Authors' contributions

JHK, MK and KYP designed the study. JHK and MK performed the experiments. JHK, MK, JMK, MKL, SJS and KYP analyzed and interpreted the data. JHK, MK, MKL, SJS and KYP prepared the manuscript. All authors read and approved the final version of this manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References