Afzelin suppresses proinflammatory responses in particulate matter-exposed human keratinocytes

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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 *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 μg/cm^2) in the presence or absence of afzelin (200 μM). 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*-α-L-rhamnopyranoside; Fig. 1A) is a flavonoid isolated from *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 μg/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)

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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% CO_2 atmosphere. At confluence, cells were treated with 0.05% trypsin/0.53 mM EDTA for 5 min at 37˚C. HaCaT cells used in subsequent experiments were between passages 20 and 24.

Cell viability assay. Cell viability was assessed using a commercial water-soluble tetrazolium salt (WST-1) assay kit (EZ-CYTOX; DoGenBio, Seoul, South Korea) according to the manufacturer's protocol. Briefly, cells were seeded at $5x10^3$ cells/well in 96-well plates. Following 24 h, cells were treated with various concentrations of PM (0, 5, 10, 25, 50 or 100 $\mu g/cm^2$) and afzelin (0, 50, 100 or 200 μM) for 24, 48 or 72 h. Following incubation, WST-1 reagent solution (10 μl) was added to each well containing 100 μl of serum-free DMEM. Plates were incubated at 37˚C for 1 h and the absorbance measured at 450 nm using a microplate spectrophotometer. Cell viability was calculated using absorbance values and data were normalized to the untreated control.

ROS assay. Intracellular ROS levels were measured by detecting the fluorescence intensity of an oxidant-sensitive dye, 2',7'-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA diffuses into cells and is deacetylated by cellular esterases to produce non-fluorescent DCFH, which rapidly oxidizes to highly fluorescent 2',7'-dichlorodihydrofluorescein (DCF) (24). Cells were seeded at $5x10^3$ cells/well in black 96-well plates. Following 24 h, cells were incubated with afzelin (200 μM in ethanol) for 2 h prior to exposure to PM (25 $\mu g/cm^2$) in serum-free DMEM for 2 h. In another experiment, following the initial 24 h culturing, cells were incubated with PM (25 $\mu g/cm^2$) for 10 min prior to the addition of afzelin (200 μM) and incubation for 2 h. Single treatment controls were established by incubation with PM (25 $\mu g/cm^2$) or afzelin (200 μM) for 2 h. Medium was removed and cells were incubated with 20 μM DCFH-DA for 30 min in the dark at 37˚C. Fluorescence intensity was detected at 485 nm excitation and 535 nm emission wavelengths. Fluorescence intensity was recorded every 15 min for 2 h for the PM only treatment group or at 2 h post PM treatment for all other treatment groups. Relative intracellular ROS fluorescence intensity was normalized by subsequent staining with PI (stock, 1 mg/ml; dilution, 1:200) and measurement of PI (propidium iodide) fluorescence. Intensity was calculated relative to the untreated control.

RNA 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 $\mu g/cm^2$) for 2 h using TRIzol reagent (Welgene, 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 $2^{-\Delta\Delta Cq}$ method (25). Primer sequences were as follows: IL-1α forward, 5'-ATCAGTACCTCACGGCTGCT-3' and reverse, 5'-TGGGTATCTCAGGCATCTCC-3'; IL-1β forward, 5'-GGGCCTCAAGGAAAAGAATC-3' and reverse, 5'-TTC TGCTTGAGAGGTGCTGA-3'; IL-6 forward, 5'-GCCTTC GGTCCAGTTGCCTT-3' and reverse, 5'-GCAGAATGAGAT GAGTTGTC-3'; and GAPDH forward, 5'-GAGTCAACGGAT TTGGTCGT-3' and reverse, 5'-TTGATTTTGGAGGGATCT CG-3'.

Western blot analysis. Cells ($1.3x10^6$/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 $\mu g/cm^2$) 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 ($1.3x10^6$/well) were cultured in 6-well plates and were pretreated with afzelin (200 μM) for 24 h followed by incubation with PM (25 $\mu g/cm^2$) 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

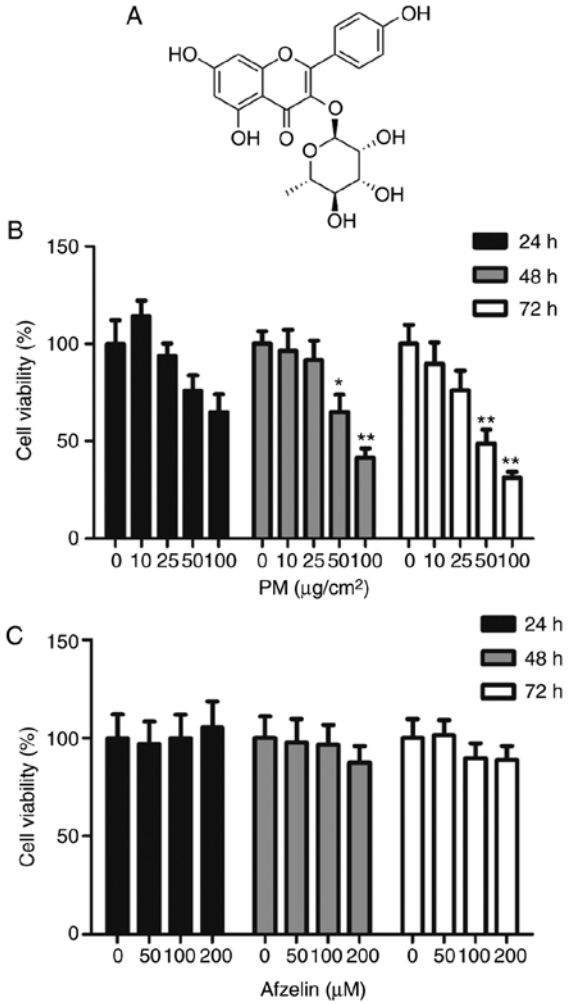

Figure 1. Effects of PM and afzelin on HaCaT viability. (A) Chemical structure of afzelin. Viability of HaCaT cells treated with (B) PM (0, 10, 25, 50 or 100 μg/cm^2) or (C) afzelin (0, 50, 100 or 200 μM) for 24, 48 or 72 h measured using the water-soluble tetrazolium salt-1 assay. The ratio of surviving cells is relative to the untreated control. Data are presented as the mean ± standard (n=3). *P<0.05, **P<0.01 vs. untreated control. PM, particulate matter.

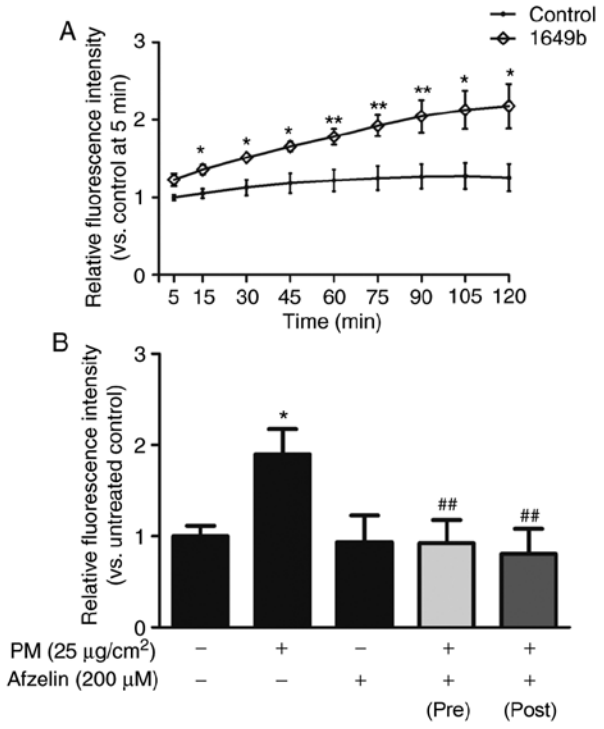

Figure 2. ROS-scavenging effect of afzelin on HaCaT cells following PM treatment. (A) Relative intracellular ROS fluorescence intensity in HaCaT cells the presence of PM (25 μg/cm^2). Measurements were performed every 15 min for 2 h. Fluorescence intensity was calculated relative to the control at 5 min. (B) Relative intracellular ROS fluorescence intensity at 2 h following treatment; cells were treated with afzelin (200 μM) or PM (25 μg/cm^2) for 2 h or incubated with PM (25 μg/cm^2) 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.

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/cm^2) and afzelin (50, 100 or 200 μM) for 24, 48 or 72 h. Cell viability was determined using the WST-1 assay. At 48 and 72 h, significant decreases in viability were observed for PM at 50 and 100 μg/cm^2 compared with the untreated control (Fig. 1B). Therefore, subsequent experiments were performed with 25 μg/cm^2 PM. As presented in Fig. 1C, afzelin not significantly affected cell viability at ≤200 μM over 24, 48 or 72 h. The results indicated that afzelin at the tested concentrations exhibited no cytotoxicity towards HaCaT cells.

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/cm^2) 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/cm^2). 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/cm^2) 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

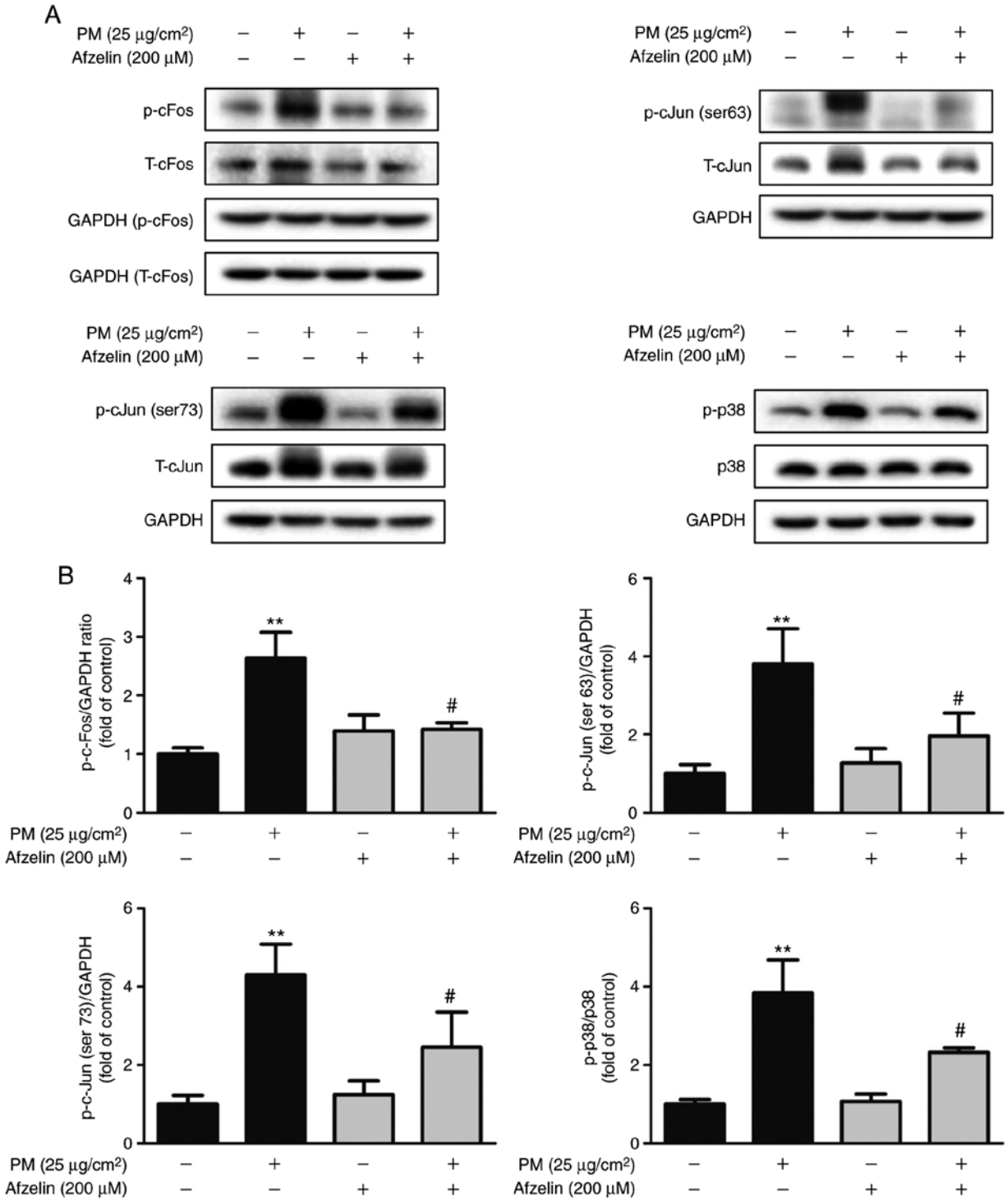

Figure 3. Effects of afzelin on transcription factor activator protein-1 components and p38 MAPK phosphorylation in HaCaT cells following PM treatment. Protein levels of p38 MAPK, c-Jun (serine 63 and 73) and c-Fos were analyzed by western blotting. (A) Western blot images for total and phosphorylated proteins of cells treated with PM (25 μg/cm^2) 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.

PM-induced proinflammatory cytokine expression, HaCaT cells were pretreated with afzelin (200 μM) for 24 h followed by PM (25 μg/cm^2) 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).

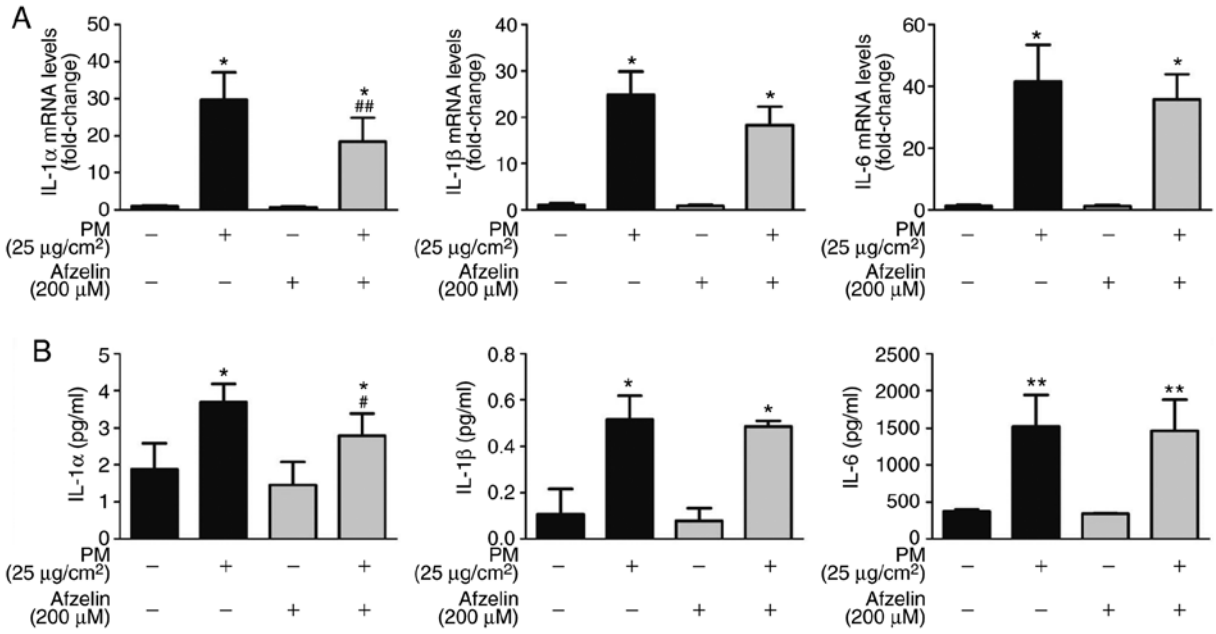

Figure 4. Effects of afzelin on mRNA and protein levels of proinflammatory cytokines in HaCaT cells following PM treatment. (A) Relative mRNA levels of IL-1α, IL-1β and IL-6 in HaCaT cells following 24 h pretreatment with afzelin (200 μM) and 2 h treatment with PM (25 μg/cm^2). Expression was normalized to GAPDH. (B) Protein levels of IL-1α, IL-1β and IL-6 determined using ELISA. Cells were pretreated with afzelin (200 μM) for 24 h and subsequently incubated with PM (25 μg/cm^2) for 24 h; samples were normalized to standard curves. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01 vs. untreated control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. PM-treated. PM, particulate matter; IL, interleukin.

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/cm^2 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 in PM-treated HaCaT cells. Production 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.

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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

1. Kim KE, Cho D and Park HJ: Air pollution and skin diseases: Adverse effects of airborne particulate matter on various skin diseases. Life Sci 152: 126-134, 2016.
2. Mancebo SE and Wang SQ: Recognizing the impact of ambient air pollution on skin health. J Eur Acad Dermatol Venereol 29: 2326-2332, 2015.
3. Magnani ND, Muresan XM, Belmonte G, Cervellati F, Sticozzi C, Pecorelli A, Miracco C, Marchini T, Evelson P and Valacchi G: Skin damage mechanisms related to airborne particulate matter exposure. Toxicol Sci 149: 227-236, 2016.
4. Kampa M and Castanas E: Human health effects of air pollution. Environ Pollut 151: 362-367, 2008.
5. Du Y, Xu X, Chu M, Guo Y and Wang J: Air particulate matter and cardiovascular disease: The epidemiological, biomedical and clinical evidence. J Thorac Dis 8: E8-E19, 2016.
6. Guarnieri M and Balmes JR: Outdoor air pollution and asthma. Lancet 383: 1581-1592, 2014.
7. Bickers DR and Athar M: Oxidative stress in the pathogenesis of skin disease. J Invest Dermatol 126: 2565-2575, 2006.
8. Segre JA: Epidermal barrier formation and recovery in skin disorders. J Clin Invest 116: 1150-1158, 2006.
9. Pan TL, Wang PW, Aljuffali IA, Huang CT, Lee CW and Fang JY: The impact of urban particulate pollution on skin barrier function and the subsequent drug absorption. J Dermatol Sci 78: 51-60, 2015.
10. Kim J, Kim EH, Oh I, Jung K, Han Y, Cheong HK and Ahn K: Symptoms of atopic dermatitis are influenced by outdoor air pollution. J Allergy Clin Immunol 132: 495-498.e1, 2013.
11. Vierkotter A, Schikowski T, Ranft U, Sugiri D, Matsui M, Krämer U and Krutmann J: Airborne particle exposure and extrinsic skin aging. J Invest Dermatol 130: 2719-2726, 2010.
12. Jin SP, Li Z, Choi EK, Lee S, Kim YK, Seo EY, Chung JH and Cho S: Urban particulate matter in air pollution penetrates into the barrier-disrupted skin and produces ROS-dependent cutaneous inflammatory response in vivo. J Dermatol Sci: S0923-1811: 30202-30210, 2018.
13. Lee CW, Lin ZC, Hu SC, Chiang YC, Hsu LF, Lin YC, Lee IT, Tsai MH and Fang JY: Urban particulate matter down-regulates filaggrin via COX2 expression/PGE2 production leading to skin barrier dysfunction. Sci Rep 6: 27995, 2016.
14. Krutmann J, Liu W, Li L, Pan X, Crawford M, Sore G and Seite S: Pollution and skin: From epidemiological and mechanistic studies to clinical implications. J Dermatol. Sci 76: 163-168, 2014.
15. Lee CW, Lin ZC, Hsu LF, Fang JY, Chiang YC, Tsai MH, Lee MH, Li SY, Hu SC, Lee IT and Yen FL: Eupafolin ameliorates COX-2 expression and PGE2 production in particulate pollutants-exposed human keratinocytes through ROS/MAPKs pathways. J Ethnopharmacol 189: 300-309, 2016.
16. McCubrey JA, LaHair MM and Franklin RA: Reactive oxygen species-induced activation of the MAP kinase signaling pathways. Antioxid Redox Signal 8: 1775-1789, 2006.
17. Parveen Z, Deng Y, Saeed MK, Dai R, Ahamad W and Yu YH: Antiinflammatory and analgesic activities of *Thesium chinense* Turcz extracts and its major flavonoids, kaempferol and kaempferol-3-O-glucoside. Yakugaku Zasshi 127: 1275-1279, 2007.
18. Lee IK, Kim KH, Choi SU, Lee JH and Lee KR: Phytochemical constituents of *Thesium chinense* TURCZ and their cytotoxic activities in vitro. Natural Product Sciences 15: 246-249, 2009.
19. Kim SK, Kim HJ, Choi SE, Park KH, Choi HK and Lee MW: Anti-oxidative and inhibitory activities on nitric oxide (NO) and prostaglandin E2 (COX-2) production of flavonoids from seeds of prunus tomentosa thunberg. Arch Pharm Res 31: 424-428, 2008.
20. Diantini A, Subarnas A, Lestari K, Halimah E, Susilawati Y, Supriyatna, Julaeha E, Achmad TH, Suradji EW, Yamazaki C, *et al*: Kaempferol-3-O-rhamnoside isolated from the leaves of Schima wallichii korth. inhibits MCF-7 breast cancer cell proliferation through activation of the caspase cascade pathway. Oncol Lett 3: 1069-1072, 2012.
21. Lee SY, So YJ, Shin MS, Cho JY and Lee J: Antibacterial effects of afzelin isolated from Cornus macrophylla on Pseudomonas aeruginosa, a leading cause of illness in immunocompromised individuals. Molecules 19: 3173-3180, 2014.
22. Shin SW, Jung E, Kim S, Kim JH, Kim EG, Lee J and Park D: Antagonizing effects and mechanisms of afzelin against UVB-induced cell damage. PLoS One 8: e61971, 2013.
23. Jung E, Kim JH, Kim MO, Jang S, Kang M, Oh SW, Nho YH, Kang SH, Kim MH, Park SH and Lee J: Afzelin positively regulates melanogenesis through the p38 MAPK pathway. Chem Biol Interact 254: 167-172, 2016.
24. Eruslanov E and Kusmartsev S: Identification of ROS using oxidized DCFDA and flow-cytometry. Methods Mol Biol 594: 57-72, 2010.
25. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
26. Drakaki E, Dessinioti C and Antoniou CV: Air pollution and the skin. Front Environ Sci 2: 11, 2014.
27. Tekpli X, Holme JA, Sergent O and Lagadic-Gossmann D: Importance of plasma membrane dynamics in chemical-induced carcinogenesis. Recent Pat Anticancer Drug Discov 6: 347-353, 2011.
28. Li N, Xia T and Nel AE: The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. Free Radic Biol Med 44: 1689-1699, 2008.
29. Wang J, Huang J, Wang L, Chen C, Yang D, Jin M, Bai C and Song Y: Urban particulate matter triggers lung inflammation via the ROS-MAPK-NF-κB signaling pathway. J Thorac Dis 9: 4398-4412, 2017.

30. Lobo V, Patil A, Phatak A and Chandra N: Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev 4: 118-126, 2010.
31. Ushio H, Nohara K and Fujimaki H: Effect of environmental pollutants on the production of pro-inflammatory cytokines by normal human dermal keratinocytes. Toxicol Lett 105: 17-24, 1999.
32. Choi H, Shin DW, Kim W, Doh SJ, Lee SH and Noh M: Asian dust storm particles induce a broad toxicological transcriptional program in human epidermal keratinocytes. Toxicol Lett 200: 92-99, 2011.
33. Li Q, Kang Z, Jiang S, Zhao J, Yan S, Xu F and Xu J: Effects of ambient fine particles $PM_{2.5}$ on human HaCaT cells. Int J Environ Res Public Health 14: E72, 2017.