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ORIGINAL ARTICLE

Negative Air Ions Alleviate Particulate Matter-Induced Inflammation and Oxidative Stress in the Human Keratinocyte Cell Line HaCaT

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Background: Recent studies have revealed that particulate matter induces inflammation, oxidative stress, and several skin diseases. Experimental results have also shown that negative air ions are highly effective in removing particulate matter-induced inflammation. **Objective:** The present study aimed to investigate whether negative air ions can inhibit inflammatory responses and reduce oxidative stress in HaCaT cells exposed to particulate matters. **Methods:** HaCaT cells were treated with particulate matter in the presence or absence of negative air ions and the viability was evaluated by the MTT assay. Reactive oxygen species (ROS) generation was quantified by the dichlorodihydrofluorescein diacetate assay. The expression of genes and proteins was analyzed by real-time polymerase chain reaction and Western blot. Levels of inflammatory cytokines were quantified by enzyme-linked immunosorbent assay. **Results:** Negative air ions were observed to downregulate the mRNA and protein levels of particulate matter-induced pro-inflammatory cytokines in HaCaT cells. In addition, negative air ion treatment suppressed particulate matter-induced intracellular ROS generation, p38 mitogen-activated protein kinase activation, and activator protein 1 (c-Fos and c-Jun) activation. **Conclusion:** Our findings indicate that negative air ions exert anti-inflammatory and antioxidant effects in HaCaT cells exposed to particulate matter. Therefore, negative air ions can be used for the prevention and treatment of particulate matter-related inflammatory skin diseases. **(Ann Dermatol 33(2) 116～121, 2021)**

-Keywords-
Inflammation, Negative air ions, Oxidative stress, Particulate matter, Reactive oxygen species

INTRODUCTION

Particulate matters (PMs) are widespread air contaminants that comprise a compound mixture of solid and liquid particles of diverse sizes and compositions. PM distribution increases with urbanization and industrialization[1]. PM may have many adverse effects on human health. Various studies have revealed that PM induces inflammation and oxidative stress, and exposure to PM that can penetrate and accumulate in the respiratory and cardiovascular systems may lead to the development of various diseases[2,3].

The skin is the largest organ in the human body and has the highest exposure to environmental contaminants. Several recent studies have shown that PM induces skin barrier impairment and inflammatory disorder[1,4].

In addition, PM has also been demonstrated to increase the generation of reactive oxygen species (ROS) and promote inflammation[4-9]. Increased ROS levels play an important role in DNA damage and the ROS-induced in-

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crease in oxidative stress activates mitogen-activated protein kinases (MAPKs) and transcription factor activator protein-1 (AP-1), ultimately leading to the release of pro-inflammatory cytokines[10,11].

Air ions are electrically charged molecules or atoms present in the atmosphere. While negative air ions (NAIs) are molecules that gain electrons, positive air ions lose electrons. NAIs primarily affect the cardiovascular and respiratory systems and also influence mental health. Various studies have demonstrated the beneficial or therapeutic effects of NAI exposure on lung function, metabolic measures, and asthmatic symptoms[12]. Some experimental findings have revealed that NAI exposure is associated with reduced depression severity, lower psychological stress, reduced anxiety, and enhanced well-being[13-15]. Artificially ionized air is commonly used for diminishing particle concentrations in indoor circumstances. NAIs have been reported to reduce the aerosol particles, airborne microbes, odor, and volatile organic compounds in indoor air[16]. The biological effects of air ions on indoor air quality as well as the various health advantages of air ionization, have been analyzed in detail by Grinshpun et al[17].

However, NAI effect on PM-mediated cutaneous responses remain to be obscure. Therefore, the present research aimed to evaluate whether NAIs play beneficial roles in PM-induced cell damage by reducing ROS production and downregulating pro-inflammatory factor expression.

MATERIALS AND METHODS

Materials and preparation

The negative ion generator Dermio Care® (Weyergans High Care AG, Western Rhineland, Germany) generating 4×10^5 negative ions/cm^3 was used in this study. The standard reference materials (SRMs) 1648a (pmA) and 1649b (pmB) were acquired from the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA) and dispersed in distilled water. Specific antibodies used for Western blot analysis, including anti-phospho-p38 MAPK, anti-p38 MAPK, anti-phospho-JNK MAPK, anti-JNK MAPK, anti-phospho-ERK1/2 MAPK, anti-ERK1/2 MAPK, anti-phospho-c-Fos, anti-phospho-c-Jun, anti-phospho-p65, and anti-p65 antibodies, were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and viability assay

HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere having 5% CO_2. Upon reaching confluence, the cells were treated with 0.05% trypsin in 0.53 mM EDTA and incubated for 5 minutes at 37°C. The HaCaT cells used in these experiments were between passages 20 and 24. The cells were conditioned for at least 12 hours in serum-free medium before experimentation.

Cell viability was determined using a water-soluble tetrazolium salt (WST-1) assay kit (EZ-CYTOX; Dogen, Seoul, Korea). Briefly, 5×10^3 cells were seeded in each well of a 96-well plate. After 24 hours, the cells were treated with NAI at varying incubation periods (0, 5, 10, and 20 minutes) in a humidified atmosphere having 5% CO_2 and incubated with WST-1 reagent solution at 37°C for 1 hour. The absorbance values were estimated at 450 nm using a SpectraMax340pc (Molecular Devices, Sunnyvale, CA, USA) microplate spectrophotometer. Relative cell viability was estimated based on the absorbance values and normalizing the values against those obtained for the control group.

ROS assay

Intracellular ROS levels were estimated by perceiving the fluorescence intensities of the oxidant-sensitive probe 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, DCFH-DA diffuses into cells and is deacetylated by cellular esterases to form non-fluorescent DCFH, which is then rapidly oxidized to the highly fluorescent DCF by the ROS present in the cells. Cells were seeded at a density of 5×10^3 cells/well in 96-well black plates. The medium was removed and the cells incubated with 20 μM DCFH-DA for 30 minutes in the dark at 37°C. Subsequently, the cells were washed thrice with phosphate-buffered saline (PBS) and incubated with PM in serum-free DMEM for 10 minutes. The PM-induced cells were treated with NAI for 20 minutes and subsequently incubated for an additional 2 hours. Fluorescence intensities were checked at an excitation wavelength of 485 nm and emission wavelength of 535 nm utilizing a SpectraMax340pc (Molecular Devices, Sunnyvale, CA, USA) microplate spectrophotometer.

RNA extraction and real-time quantitative PCR analysis

Total RNA was extracted from cells using the TRIzol reagent (Welgene, Seoul, Korea). To synthesize the cDNA templates, mRNAs were reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) and incubated for 1 hour at 42°C. The cDNA was amplified by real-time quantitative PCR with specific primers for interleukin (IL)-1α, IL-1β, IL-6, and GAPDH. Real-time quantitative PCR assays were conducted on a QuantStudio 3 system (Applied Biosystems, Foster City, CA, USA) using PowerUp SYBR Green Master Mix (Applied Biosystems). Expression levels were normalized to those

of the housekeeping gene, GAPDH. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method according to the manufacturer's instructions.

Western blot analysis

Western blot was conducted following standard procedures. Briefly, the cells were lysed with 1% Triton-X radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail. The remaining cell debris was pulled out by centrifugation (13,200 rpm, 15 minutes), and the protein concentration was determined by the bicinchoninic acid assay method. Identical amounts of protein were loaded on 8%~15% sodium dodecyl sulfate-polyacrylamide gels and separated by electrophoresis. After electrophoresis, the proteins were transferred on nitrocellulose membranes, which were blocked with 5% skim milk for 1 hour. Subsequently, the membranes were incubated with primary antibodies (1:1,000 dilution) overnight at 4°C, and with horseradish peroxidase-conjugated secondary antibodies (1:2,000 dilution) for 1 hour at room temperature. Proteins were detected using the EzWestLumi plus system (ATTO, Tokyo, Japan), and images obtained using a ChemiDoc™ XRS image analyzer (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All data have been presented as the mean±standard deviation. The mean values were calculated based on data from at least three independent experiment experiments, which were performed on separate days using freshly prepared reagents. Data were interpreted using the paired t-test. p-values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS (PASW statistics 18; IBM Corp., Armonk, NY, USA).

RESULTS

Cytotoxicity of particulate matters and negative air ions in HaCaT cells

Significant cytotoxicity was observed on treating with 100 $\mu g/cm^2$ pmA and >50 $\mu g/cm^2$ pmB. Therefore, 50 $\mu g/cm^2$ pmA and 25 $\mu g/cm^2$ pmB were used in the experiments. As shown in Supplementary Fig. 1, anion-treated HaCaT cells exhibited more than 90% cell viability after 5, 10, and 20 minutes of treatment. These results indicated that NAIs do not exhibit cytotoxicity toward HaCaT cells.

Intracellular ROS-scavenging effect of negative air ions in particulate matter-treated HaCaT cells

To analyze PM-induced intracellular ROS generation, HaCaT cells were treated with pmA and pmB and the intracellular ROS levels were determined by estimating the fluorescence intensity of the oxidant-sensitive probe DCFH-DA. Treatment with PMs induced a dose-dependent increase in intracellular ROS levels relative to that in the negative control group (data not shown). To assess the intracellular ROS-scavenging effect of NAI, the cells were treated with NAI for 20 minutes after PM exposure. As shown in Fig. 1, NAI insignificantly reduced pmA-induced ROS generation whereas it had no effect on pmB-induced ROS.

Inhibitory effect of negative air ions on the pro-inflammatory cytokine production in particulate matter-treated HaCaT cells

To evaluate whether NAIs diminished the PM-induced expression of pro-inflammatory cytokines IL-1 α, IL-1 β, and IL-6, HaCaT cells were treated with PMs in the presence or absence of NAIs. Real-time quantitative PCR analysis was conducted to estimate the mRNA expression levels of IL-1 α, IL-1 β, and IL-6. To confirm the results, the cells were pre-treated with PMs and subsequently treated with NAIs for 20 minutes. Results showed that PMs upregulated IL-1 α, IL-1 β, and IL-6 mRNA expressions in HaCaT cells, while NAIs downregulated IL-1 α, IL-1 β, and IL-6 expressions (Fig. 2).

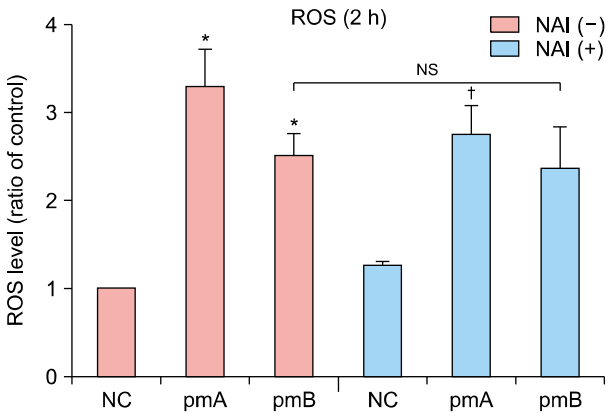

Fig. 1. NAI effectively reduced PM-induced ROS generation. The HaCaT cells were serum starved for overnight and incubated with PMs (pmA: 50 $\mu g/cm^2$; pmB: 25 $\mu g/cm^2$). After 10 minutes, cells were treated with NAI for 20 minutes and subsequently incubated for an additional 2 hours. Intracellular ROS levels were estimated by detecting the fluorescence intensity of the oxidant-sensitive florescent probe DCFH-DA. The florescence intensity was checked in the presence or absence of PM at 485 nm/535 nm. Normalizing of the ROS florescence intensity was calculated using the propidium iodide fluorescence measurement intensity ratio. NAI: negative air ions, ROS: reactive oxygen species, PM: particulate matter, NC: negative control, pmA: standard reference materials (SRMs) 1648a, pmB: SRMs 1649b, NS: no significance. *$p<0.05$ vs. NC; †$p<0.05$ vs. NAI treated NC.

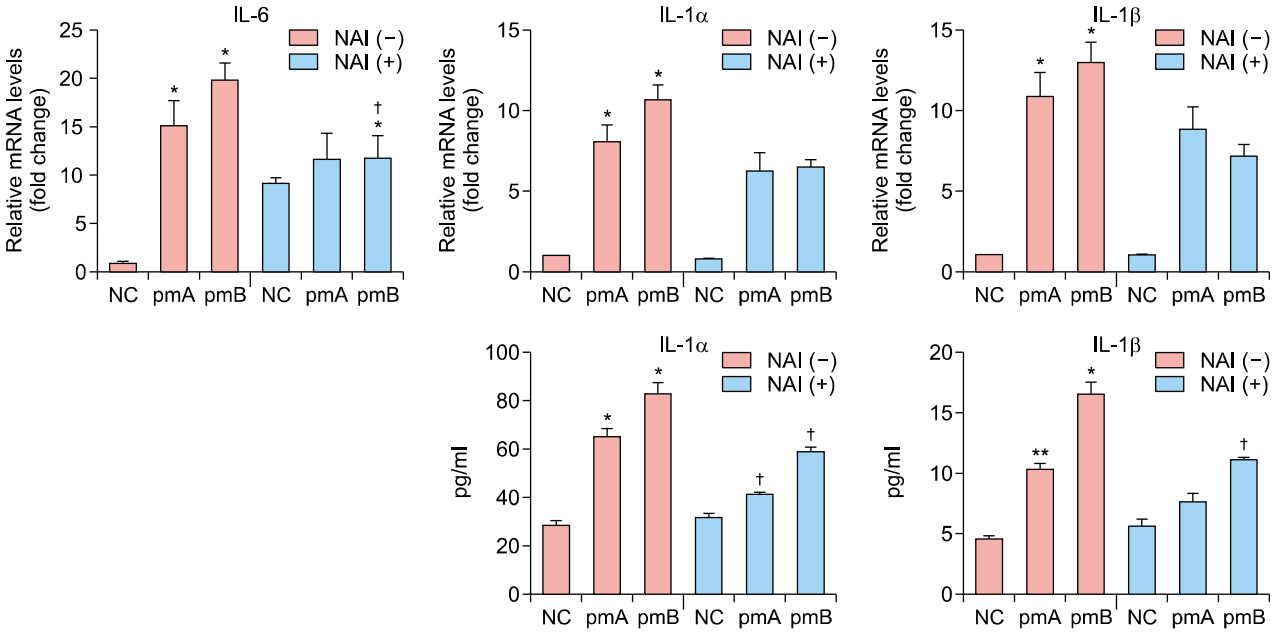

Fig. 2. Particulate matter (PM) upregulated the expression of IL-1 α, IL-1 β, and IL-6 in HaCaT cells, while NAI downregulated the mRNA and protein expression levels of IL-1 α, IL-1 β, and IL-6. The mRNA and protein expressions after treatment of PMs (pmA: 50 μg/cm^2; pmB: 25 μg/cm^2) on HaCaT cells were represented in graphical form (the relative fold change of mRNA compared with change in untreated cells). The protein expression analyzed using enzyme-linked immunosorbent assay. NAI: negative air ions, IL: interleukin, NC: negative control, pmA: standard reference materials (SRMs) 1648a, pmB: SRMs 1649b, NS: no significance. *$p<0.05$ vs. NC; **$p<0.01$ vs. NC; $^{\dagger}p<0.05$ vs. NAI untreated each PM group.

Effect of negative air ions on AP-1 and p38 protein expression in particulate matter-treated HaCaT cells

Western blot was conducted to determine the effect of NAIs on the expression levels of the signaling molecules AP-1 and p38. After pretreatment with PMs, cells were treated with NAIs for 20 minutes. Treatment with NAIs downregulated PM-induced phosphorylation levels of p38 and JNK MAPKs and p65. In addition, the protein levels of c-Fos and c-Jun, the components of the transcription factor AP-1, were also suppressed (Fig. 3).

DISCUSSION

Recent researches have evaluated the effects of PM on skin impairment and determined the basal molecular mechanisms. Air pollution including PM was realized to elevate the prevalence of inflammatory skin disorder and skin cancer[1,18]. Studies indicated that these diseases are mediated by ROS generation, inflammatory cytokine production, DNA repair system impairment, and aryl hydrocarbon receptor, MAPK (ERK1/2, JNK, and p38), transcription factor (NF-κB and AP-1), and matrix metalloproteinase promoting collagen degradation activation[8-11].

We investigated whether NAIs suppress the inflammatory responses and reduce oxidative stress in PM-exposed HaCaT cells. Diverse pollutants can induce ROS production and the elevated ROS levels play a role in the pathogenesis of human skin diseases and the impairment of function[1,4]. Urban PM containing environmental hormones, incomplete combustion products, and heavy metals are more toxic to skin cells. Therefore, the SRMs issued by the NIST including SRM 1648a (pmA; used as the urban particulate matter NIST index) and SRM 1649b (pmB; used as the urban dust NIST index) mainly comprising heavy metals and polycyclic aromatic hydrocarbons (PAHs), respectively, were used for this research. PM containing PAHs and organic carbon contents increase oxidative stress by contributing to mitochondrial injury[19]. As air pollution has grown, especially in the Far East countries such as China, Korea, and Japan, interest in the health problems induced by PM is growing. The general public has been opting for more natural and safer treatment and preventive options such as complementary and alternative medicine as possible adjunctive PM therapies. Therefore, it would be valuable to discover a novel alternative treatment option without side effects or contraindications that is safe, effective, and can be administered to all patients who are PM-exposed. A previous study has shown that rats exposed to an atmosphere enriched with NAI for 4 weeks showed lower ma-

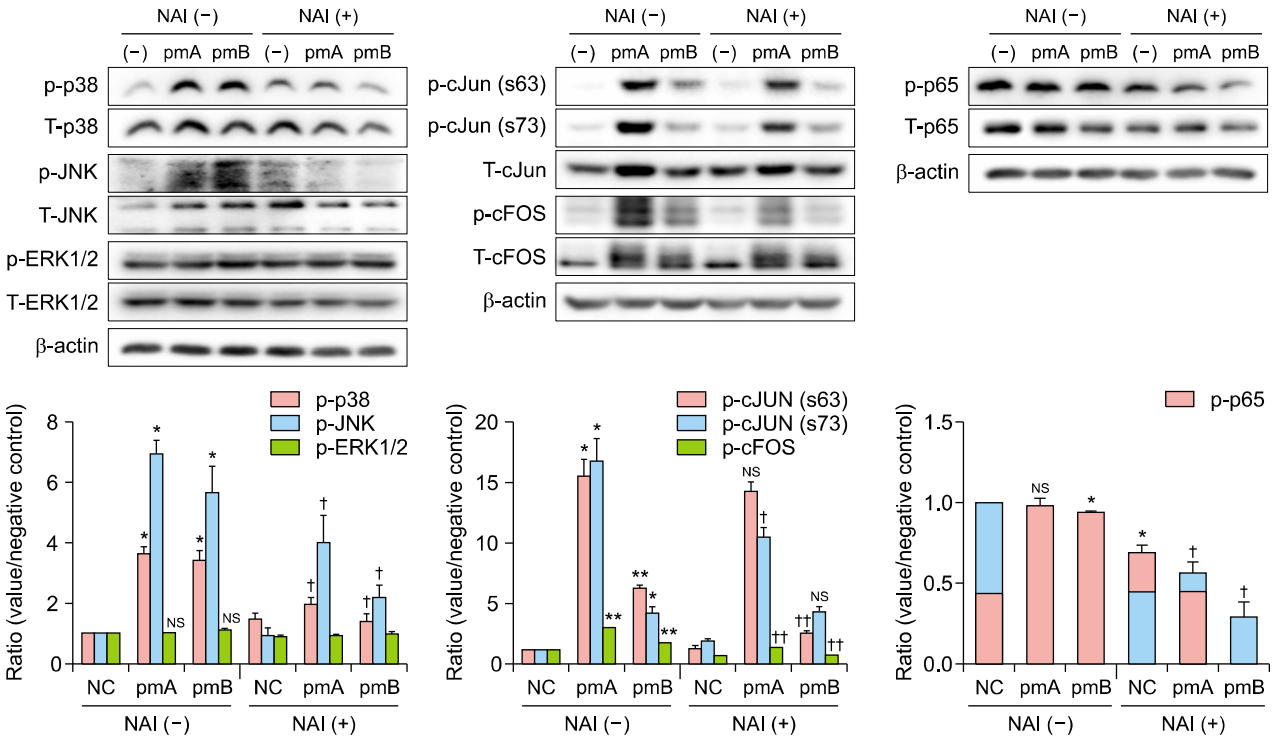

Fig. 3. NAI downregulated particulate matter (PM)-induced phosphorylation levels of p38, JNK and p65 and suppressed protein levels of c-Fos and c-Jun, the components of the transcription factor activator protein-1. HaCaT cells were treated with PMs before being treated with NAI. The protein expression levels were analyzed by Western blot analysis and represented in graphical form (the relative fold change of compared with change in untreated cells). NAI: negative air ions, NC: negative control, pmA: standard reference materials (SRMs) 1648a, pmB: SRMs 1649b, NS: no significance. *$p<0.05$ vs. NC; **$p<0.01$ vs. NC; $^{\dagger}p<0.05$ vs. NAI untreated each PM group; $^{\dagger\dagger}p<0.01$ vs. NAI untreated each PM group.

londialdehyde (as an oxidative stress biomarker) levels compared to control rats[20]. Other animal studies have shown that the ROS levels are reduced in the urine, brain, and blood upon exposure to 10,000/cm^3 NAIs for one week[21]. The characteristics of NAIs could induce active oxygen species; however, NAIs mediate deoxidation rather than oxidation by supplying electrons to the body[22]. In the present study, NAIs insignificantly attenuated intracellular ROS generation in PM-treated HaCaT cells; however, the ROS content was insignificantly increased in control group after NAI exposure (Fig. 1). Our findings indicated that NAIs may defend skin keratinocytes from PM-induced oxidative stress but cause mild intracellular ROS generation due to the characteristics of NAI-induced active oxygen species. A variety of studies have shown that air pollutants increase the production of pro-inflammatory cytokines and suggested the potential role of keratinocytes in skin inflammatory responses by releasing pro-inflammatory cytokines. Our current findings showed that NAIs reduced PM-induced p38 MAPK and AP-1 phosphorylation, which is related with the regulation of the release of the pro-inflammatory cytokines. Our findings showed that NAIs decreased the phosphorylation of p38 MAPK and AP-1 proteins (Fig. 3). In addition, NAIs diminished the release of pro-inflammatory cytokines such as IL-1α, IL-1β, and IL-6 in PM-treated HaCaT cells (Fig. 2). Our results also suggested that the expression levels of pro-inflammatory cytokines regulated by the AP-1 pathway are reduced by NAIs by inhibiting the ROS/p38 MAPK/AP-1 pathway.

In conclusion, our results displayed the anti-inflammatory effects of NAIs in PM-exposed HaCaT cells indicating that NAIs exert preventive or therapeutic effects against various skin diseases that are induced and exacerbated by PMs.

SUPPLEMENTARY MATERIALS

Supplementary data can be found via http://anndermatol.org/src/sm/ad-33-116-s001.pdf.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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