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Niacinamide Protects Skin Cells from Oxidative Stress Induced by Particulate Matter

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Abstract

Niacinamide (NIA) is a water-soluble vitamin that is widely used in the treatment of skin diseases. Moreover, NIA displays antioxidant effects and helps repair damaged DNA. Recent studies showed that particulate matter 2.5 ($PM_{2.5}$) induced reactive oxygen species (ROS), causing disruption of DNA, lipids, and protein, mitochondrial depolarization, and apoptosis of skin keratinocytes. Here, we investigated the protective effects of NIA on $PM_{2.5}$-induced oxidative stress in human HaCaT keratinocytes. We found that NIA could inhibit the ROS generation induced by $PM_{2.5}$, as well block the $PM_{2.5}$-induced oxidation of molecules, such as lipids, proteins, and DNA. Furthermore, NIA alleviated $PM_{2.5}$-induced accumulation of cellular Ca^{2+}, which caused cell membrane depolarization and apoptosis, and reduced the number of apoptotic cells. Collectively, the findings show that NIA can protect keratinocytes from $PM_{2.5}$-induced oxidative stress and cell damage.

Key Words: Niacinamide, Particulate matter 2.5, Oxidative stress, Human HaCaT keratinocyte

INTRODUCTION

Niacinamide (NIA), also known as nicotinamide, is a hydrophilic amide of vitamin B3 that is an important component in various cosmetics and medicines. NIA is found in a wide array of foods such as fish, mushroom, and nuts (Damian, 2010). As a typical medicine for treating pigmentary disorders, NIA blocks the melanosome migration between melanocytes and keratinocytes and suppresses skin pigmentation (Bissett *et al*., 2007; Rolfe, 2014). The biological activities of NIA also include antimicrobial, photo-protection, lighting, and anti-pruritus (Wohlrab and Kewft, 2014). Moreover, as a coenzyme in the glycolysis pathway, NIA promotes the repair of DNA damage induced by UV in keratinocytes (Sousa *et al*., 2012; Surjana *et al*., 2013).

Due to excessive consumption of fossil fuels, air pollution has become a major health hazard for humans (Park *et al*., 2018). Both indoor and outdoor particulate matter (PM) damage human systems, such as bronchial epithelial cells (Yuan *et al*., 2019), the cardiovascular system (Cao *et al*., 2016), the central nervous system (Wang *et al*., 2017), and the pulmonary immune system (Li *et al*., 2017). In addition, PM can penetrate the skin barrier, mainly by appendageal route and stratum corneum, thereby disrupting the protective activity of the skin (Pan *et al*., 2015), causing wrinkling and thickening (Kim *et al*., 2016). PM contributes to skin aging by inducing oxidative stress and inflammation (Magnani *et al*., 2016). We have previously reported that PM induces oxidative stress, promotes apoptosis, and causes dysfunction of keratinocytes (Piao *et al*., 2018; Zhen *et al*., 2018, 2019).

In this study, we focused on particulate matter <2.5 μm ($PM_{2.5}$), which can attach to epidermal skin due to its small size. $PM_{2.5}$ is known to stimulate the generation of reactive oxygen species (ROS) in keratinocytes (Hyun *et al*., 2019b). NIA has been shown to suppress oxidative stress notably *in vivo* (Abdullah *et al*., 2018). However, there are no reports on the cytoprotective action of NIA against $PM_{2.5}$-induced oxidative stress in human keratinocytes. Therefore, in this study, we examined whether NIA can protect HaCaT cells against $PM_{2.5}$-induced damage.

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MATERIALS AND METHODS

Preparation of NIA and $PM_{2.5}$

NIA (Fig. 1A) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA) and dissolved in dimethylsulfoxide (DMSO). Diesel particulate matter NIST SRM 1650b ($PM_{2.5}$, Sigma-Aldrich) was dissolved in DMSO and stored at a concentration of 25 mg/mL (Piao *et al*., 2018).

Cell culture

HaCaT human keratinocytes from Cell Lines Service (Heidelberg, Germany) were cultured in Dulbecco's Modified Eagle's Medium (Life Technologies Co., Grand Island, NY, USA) with 10% heat-inactivated fetal calf serum at 37°C with 5% CO_2.

Cell viability

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to test the cytotoxicity of NIA. Cells were seeded into a 24-well plate with 12.5, 25, 50, 100, or 200 μM of NIA. Then, MTT solution was added to each well and samples were incubated for 4 h. Finally, the solutions from DMSO and yield formazan crystals were detected at 540 nm using the scanning multi-well spectrophotometer (Piao *et al*., 2017).

Determination of intracellular ROS

To investigate anti-oxidative stress effect of NIA, we used 2′,7′-dichlorofluorescein diacetate (DCF-DA, Sigma-Aldrich) staining. ROS generation was induced using $PM_{2.5}$ (50 μg/mL). After 16 h of incubation, the cells were treated with 12.5, 25, 50, 100, or 200 μM of NIA and $PM_{2.5}$, followed by addition of 25 μM DCF-DA. The fluorescence of DCF-DA was measured using a flow cytometer (Becton Dickinson, Mountain View, CA, USA) (Piao *et al*., 2018). The inhibitory effect of NIA (100 μM) on $PM_{2.5}$-induced ROS was assessed by a confocal microscope (Carl Zeiss, Oberkochen, Germany).

NADP/NADPH assay

To determine the ratio of intracellular NADP and NADPH, we used NADP/NADPH assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Measurement of intracellular superoxide levels by dihydroethidium (DHE) oxidation

To detect superoxide levels in cells, we measured the DHE oxidation. Cells were treated with NIA and $PM_{2.5}$ and incubated with DHE (10 μM) for 30 min. After incubation, the fluorescence intensity was analyzed by a confocal microscope.

Lipid peroxidation assay

Lipid oxidative stress was investigated using two methods. The cells were dyed with 5 μM diphenyl-1-pyrenylphosphine (DPPP, Sigma-Aldrich) and fluorescence was analyzed using the confocal microscope. Harvested cells were subjected to analysis using the 8-Isoprostane ELISA kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions (Zhen *et al*., 2019).

Protein carbonylation assay

The harvested cells were used for detecting protein oxidation with an Oxiselect™ Protein Carbonyl ELISA kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's instructions.

Detection of 8-oxoguanine (8-oxoG)

To quantify oxidative DNA damage, we determined the level of 8-oxoG through the Bioxytech 8-OHdG ELISA kit (OXIS Health Products, Portland, OR, USA) according to the manufacturer's instructions. For image analysis, the cells were fixed on a chamber slide and stained with avidin-tetramethylrhodamine isothiocyanate (TRITC) (1:200) conjugate (Sigma-Aldrich), and fluorescence images were obtained using a confocal microscope (Piao *et al*., 2011).

Single cell gel electrophoresis (Comet assay)

The comet assay was used to detect DNA damage induced by $PM_{2.5}$. Harvested cells were dispersed in low-melting agarose (1%). Then, the mixtures were solidified on microscopic slides, and the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 1% Trion X-100, and 10% DMSO, pH 10) for 1 h at 4°C. After electrophoresis, the slides were stained with ethidium bromide, and the percentage of the comet tail fluorescence and the tail length (50 cells per slide) was determined using a fluorescence microscope equipped with an image analysis software (Kinetic Imaging, Komet 5.5, Liverpool, UK) (Park *et al*., 2017).

Quantification of Ca^{2+} level

To determine the intracellular Ca^{2+} in viable cells, the cells were co-cultured with 10 μM fluo-4-acetoxymethyl ester (Fluo-4-AM, Sigma-Aldrich) dye for 30 min, and green fluorescence in the confocal micrographs was quantified.

Mitochondrial membrane potential ($\Delta\psi_m$) analysis

To visualize the changes in membrane potential, the cells were stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylben

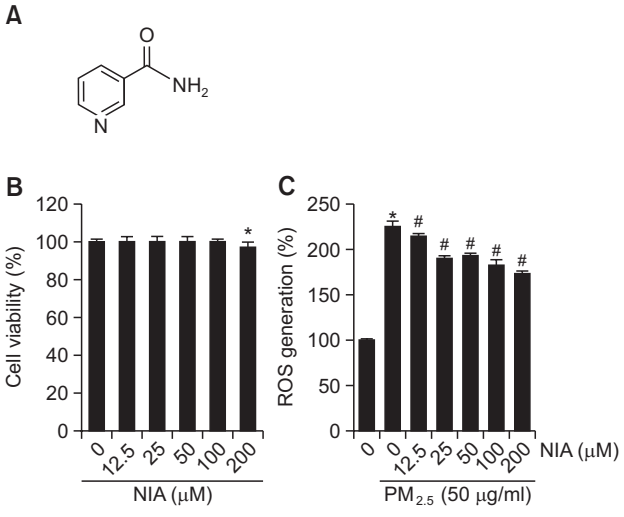

Fig. 1. Niacinamide (NIA) decreased $PM_{2.5}$-induced ROS generation. (A) Chemical structure of NIA. (B) MTT assay to determine cell viability after treatment with indicated concentrations of NIA (0, 12.5, 25, 50, 100, or 200 μM). (C) Flow cytometric determination of intracellular ROS scavenging activity of NIA (0, 12.5, 25, 50, 100, or 200 μM). ROS generation was induced by $PM_{2.5}$. $^{*}p<0.05$ and $^{\#}p<0.05$ compared with control cells and $PM_{2.5}$-exposed cells, respectively.

zimidazolylcarbocyanine iodide (JC-1, Invitrogen, Carlsbad, CA, USA) and were analyzed by confocal microscopy.

Hoechst 33342 staining

To observe apoptotic bodies, we used the nuclear-specific dye, Hoechst 33342 (Sigma-Aldrich). Cells were pre-treated with 100 μM NIA, treated with $PM_{2.5}$ for 24 h, and then stained with Hoechst 33342 (10 μM). Nuclei were visualized under a fluorescence microscope equipped with a Cool SNAP-Pro color digital camera (Media Cybernetics, Silver Spring, MD, USA) (Han *et al*., 2019).

Statistical analysis

Data are shown as mean ± standard error, and were analyzed using the Tukey's test and analysis of variance (ANOVA) by the Sigma Stat (v12) software (SPSS, Chicago, IL, USA). *p*-values <0.05 were considered statistically significant.

RESULTS

NIA showed anti-oxidative effect

Here, we used $PM_{2.5}$ at a concentration of 50 μg/mL for ROS induction. This concentration was selected based on the findings of our recent study investigating whether $PM_{2.5}$ induced oxidative stress. In that study, we measured ROS generation at various concentrations (25-100 μg/mL) of $PM_{2.5}$, and 50 μg/mL $PM_{2.5}$ was found to be optimal concentration

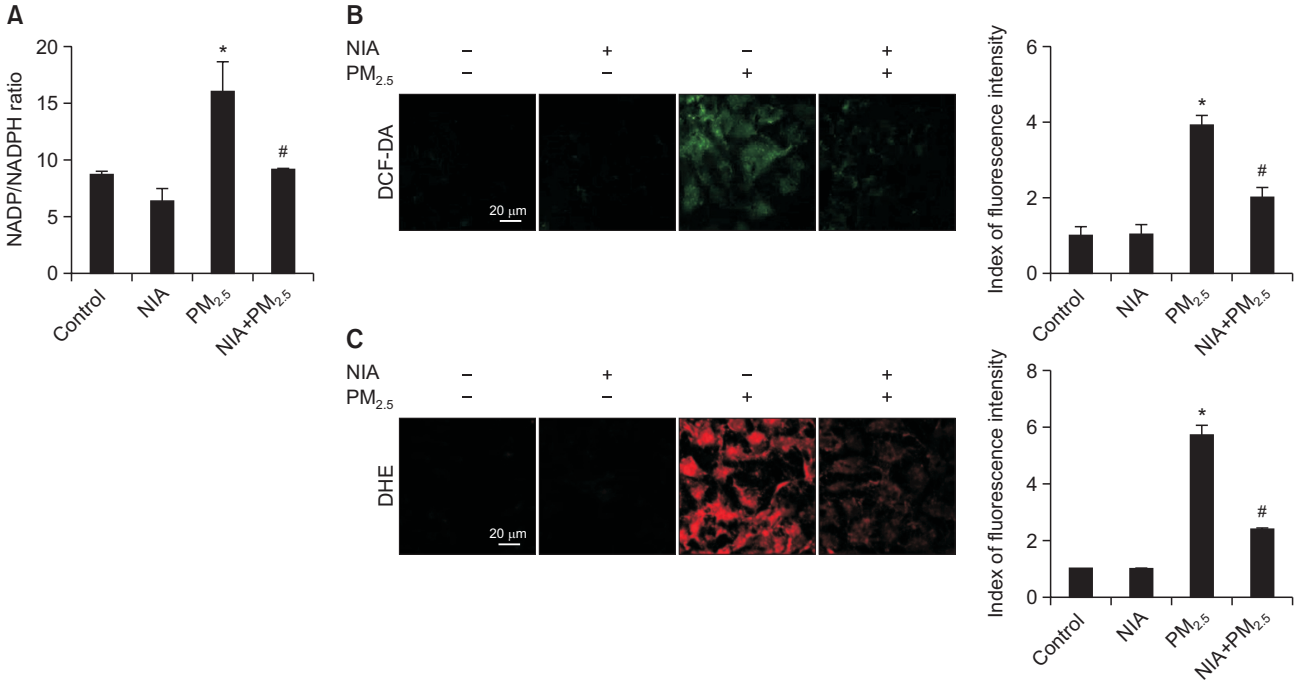

Fig. 2. NIA cleared ROS by inhibiting NOX activity induced by $PM_{2.5}$. (A) The ratio of intracellular NADP and NADPH was assessed using NADP/NADPH assay kit. (B) Intracellular ROS was detected after staining of cells with DCF-DA dye. (C) Superoxide generation was detected after dying cells with DHE. NIA diminished superoxide levels induced by $PM_{2.5}$. **p*<0.05 and $^{\#}p$<0.05 compared with control cells and $PM_{2.5}$-exposed cells, respectively.

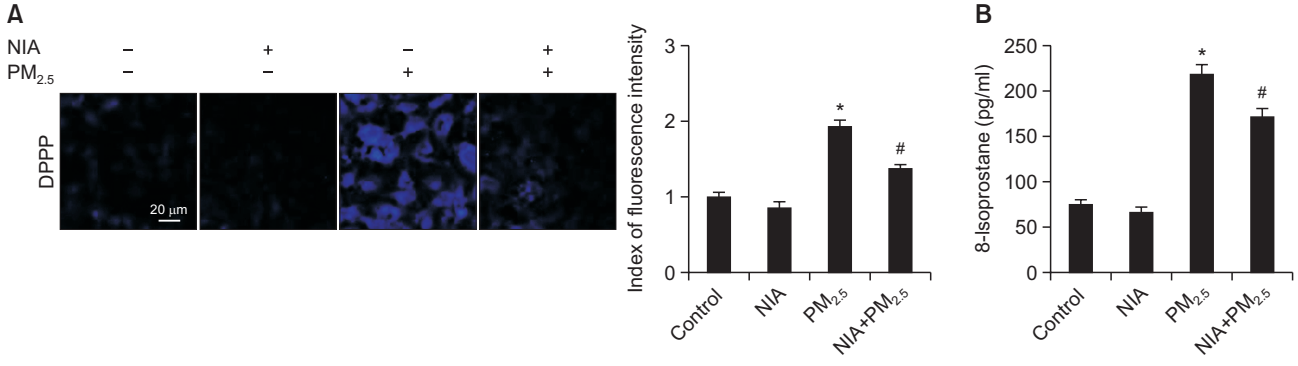

Fig. 3. NIA protected lipids from $PM_{2.5}$-induced oxidation. (A) 8-Isoprostane ELISA assay and (B) DPPP staining were used to analyze lipid peroxidation. **p*<0.05 and $^{\#}p$<0.05 compared with control cells and $PM_{2.5}$-exposed cells, respectively.

to clearly observe oxidative stress-induced cell damage and inflammatory response (Piao *et al*., 2018; Ryu *et al*., 2019). In the MTT assay, NIA, up to 100 μM, showed no toxicity toward human keratinocytes, but cytotoxicity was observed at 200 μM (Fig. 1B). In addition, NIA dose-dependently inhibited the ROS generation induced by $PM_{2.5}$ (Fig. 1C). To further investigate the anti-oxidative effect of NIA, we used 100 μM NIA as the optimal concentration for subsequent experiments.

NIA blocked ROS generation via NADPH oxidase activity

Evaluation of the NADPH oxidase (NOX) activity (NADP/NADPH ratio) showed that $PM_{2.5}$ increased the oxidation of NADPH, which was reversed by NIA pre-treatment (Fig. 2A). In the DCF-DA staining assay, NIA inhibited $PM_{2.5}$-induced fluorescence in the cells, demonstrating that NIA protected cells from $PM_{2.5}$-induced ROS generation (Fig. 2B). Similarly, DHE staining revealed that superoxide generation induced by $PM_{2.5}$ was blocked by NIA (Fig. 2C). These results further indicated that NIA exerted antioxidant effects in keratinocytes.

NIA inhibited $PM_{2.5}$-induced lipid peroxidation

The detection of phosphine oxide (DPPP oxide) using the DPPP probe indicated that $PM_{2.5}$ significantly induced lipid peroxidation, and pretreatment with NIA decreased the fluorescence emitted by the probe (Fig. 3A). Moreover, the levels of 8-isoprostane, a specific indicator of lipid peroxidation, revealed that NIA suppressed $PM_{2.5}$-induced peroxidation (Fig. 3B). These results showed that NIA rescued cells from $PM_{2.5}$-induced lipid oxidation.

NIA suppressed $PM_{2.5}$-induced oxidative stress on proteins

The levels of protein carbonylation, a specific indicator of protein oxidation, revealed that NIA significantly inhibited $PM_{2.5}$-induced oxidative protein modification (Fig. 4). These results showed that NIA rescued cells from $PM_{2.5}$-induced protein carbonylation.

NIA protected cells from $PM_{2.5}$-induced DNA damage

The 8-OHdG assay was used to assess oxidative damage on DNA caused by $PM_{2.5}$ (Fig. 5A). Notably, NIA reduced 8-OHdG generation induced by $PM_{2.5}$. Furthermore, confocal microscopy showed that the level of 8-oxoG in $PM_{2.5}$-exposed cells was the highest, suggesting severe DNA lesions formed via avidin-TRITC binding. Consistent with other results, NIA ameliorated the DNA lesions (Fig. 5B). The protective effect

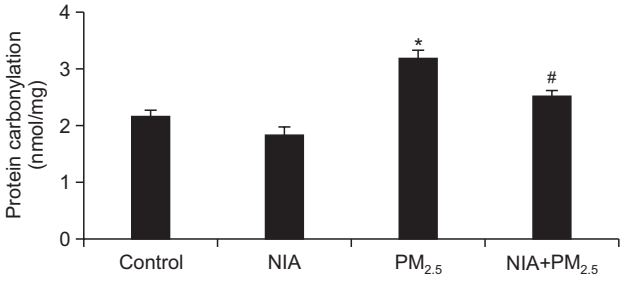

Fig. 4. NIA protected proteins from $PM_{2.5}$-induced oxidative stress. Protein oxidation was evaluated by protein carbonylation level. *p<0.05 and #p<0.05 compared with control cells and $PM_{2.5}$-exposed cells, respectively.

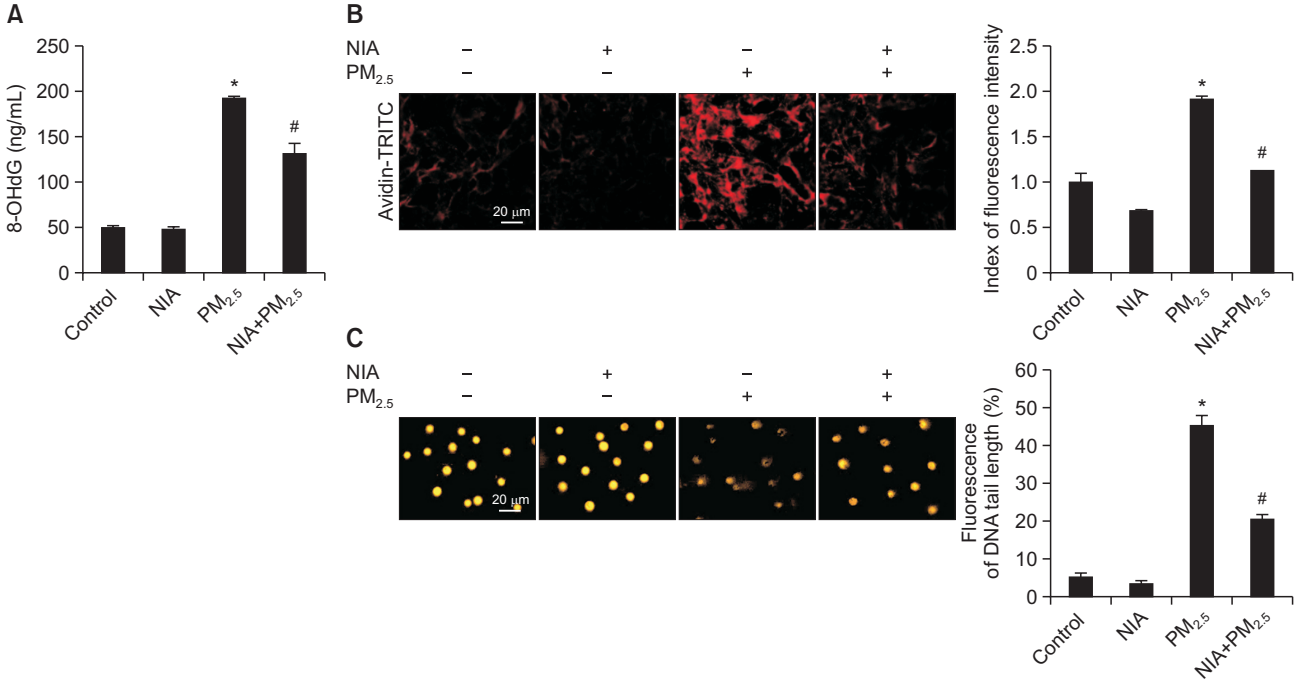

Fig. 5. NIA protected DNA from $PM_{2.5}$-induced oxidative damage. (A) 8-OHdG ELISA (Bioxytech) kit was used to measure the level of 8-OHdG. (B) Avidin-TRITC conjugate was examined to evaluate the DNA oxidative adducts of 8-oxoG using confocal microscopy. (C) Comet assay was used to detect $PM_{2.5}$-induced DNA damage. *p<0.05 and #p<0.05 compared with control cells and $PM_{2.5}$-exposed cells, respectively.

of NIA on DNA damage was also observed in the comet assy. Length of comet tails and the percentage of tail fluorescence were increased by $PM_{2.5}$, but decreased following NIA pretreatment (Fig. 5C). Collectively, these results illustrated that NIA protected against DNA damage induced by $PM_{2.5}$.

NIA lowered excessive intracellular Ca^{2+}, balanced membrane potential, and suppressed apoptosis induced by $PM_{2.5}$

Intracellular Ca^{2+}, tracked by Fluo-4-AM, was detected by confocal microscopy. The image analysis revealed that $PM_{2.5}$ stimulated excessive Ca^{2+}, which could be reduced by NIA treatment (Fig. 6A). JC-1 staining was used to determine $\Delta\psi_m$, with red and green fluorescence representing polarization and depolarization, respectively. The images obtained from confocal microscopy showed that $\Delta\psi_m$ polarization and depolarization were decreased and increased by $PM_{2.5}$ treatment, respectively; however, they were reversed by NIA pretreatment (Fig. 6B). $PM_{2.5}$ also promoted apoptotic bodies, when observed using Hoechst 33342 staining, and NIA pretreatment reduced their numbers (Fig. 6C). These results proved that $PM_{2.5}$ disrupted the homeostasis of intracellular Ca^{2+} levels and accelerated cell apoptosis, but NIA exerted cytoprotective effects against these $PM_{2.5}$-induced damages.

DISCUSSION

The skin is the outermost organ and acts as the first protective layer from air pollution. Current studies indicate that air pollutants damage skin via two main routes. The first route is from the outside to inside, whereby PM penetrates the skin

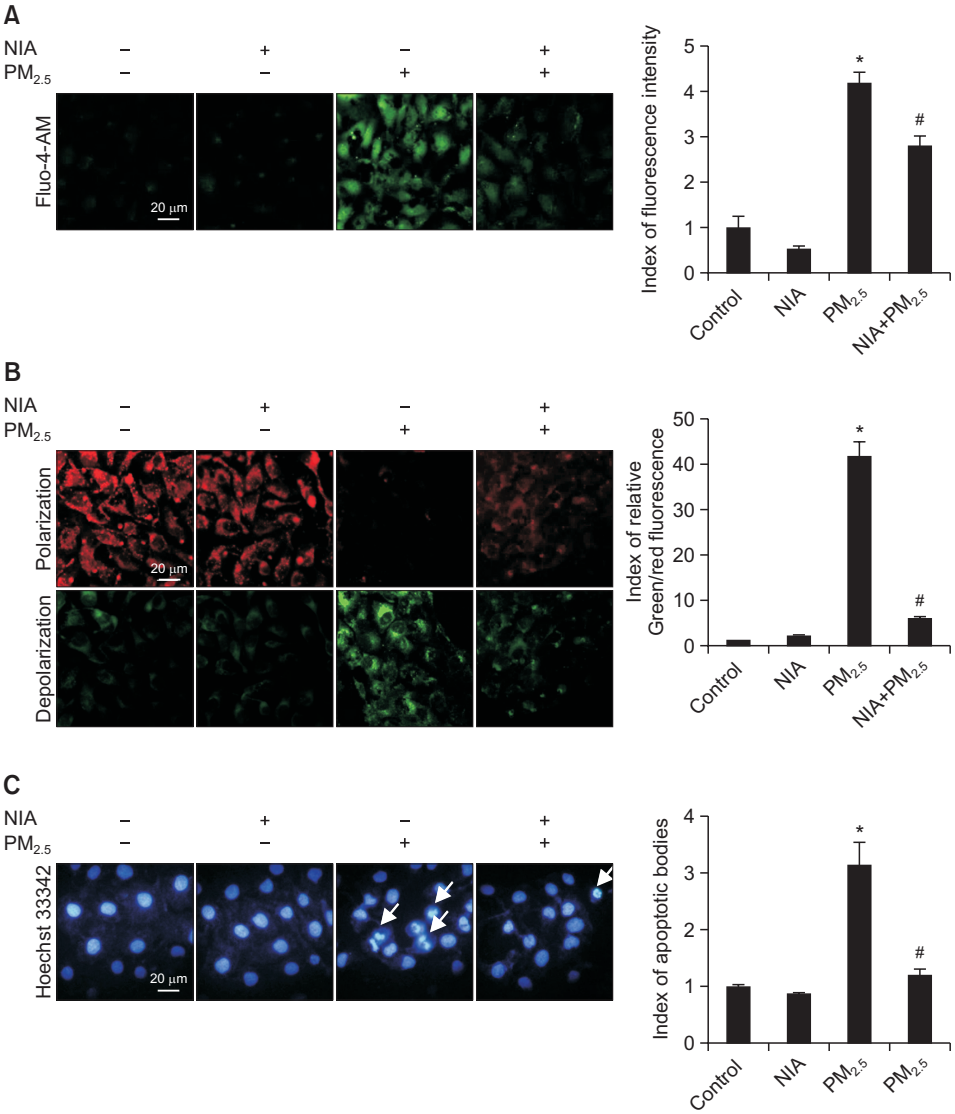

Fig. 6. NIA protected cells from $PM_{2.5}$-induced apoptosis, by blocking calcium channel and balancing mitochondrial membrane potential. (A) Fluo-4-AM staining was used to determine the intracellular Ca^{2+} levels. (B) $\Delta\psi_m$ analysis was performed using JC-1 dye and confocal microscopy. (C) Apoptotic bodies (indicated with arrows) were visualized using Hoechst 33342 dye. *p<0.05 or $^{\#}p$<0.05 compared with control cells or $PM_{2.5}$-exposed cells, respectively.

(including keratinocytes), and the second route is from the inside to the outside, whereby toxic effects in the lung subsequently influence the skin (Krutmann *et al*., 2014). In addition, $PM_{2.5}$ stimulates inflammation by disrupting the cytokine network, thereby causing epidermal hyperplasia (Kim *et al*., 2017). Moreover, PM is known to cause oxidative damage to keratinocytes by promoting ROS generation (Romani *et al*., 2018). NIA is a well-known skin whitening agent (Hakozaki *et al*., 2002) and is known to improve skin barrier function and decrease sebum secretion (Draelos *et al*., 2005). In the present study, we mainly focused on the potential protective effect of NIA on $PM_{2.5}$-induced oxidative damage in keratinocytes. NIA pretreatment at all tested concentrations inhibited $PM_{2.5}$-induced ROS generation in cells.

A previous study showed that ROS is involved in various biological processes, including oxygen sensing, cell growth, cell differentiation, and cell death (Touyz *et al*., 2019). NOX plays a vital role in intracellular superoxide and hydrogen peroxide production (Cachat *et al*., 2015) and can be induced by particle exposure in keratinocytes (Kampfrath *et al*., 2011; Sticozzi *et al*., 2012). NOXs are widely distributed in mammalian tissues and, thereby, serve various functions (Touyz *et al*., 2019). The six homologs of the cytochrome subunit of the NOX (NOX1, NOX3, NOX4, NOX5, DUOX1, and DUOX2) are predominantly responsible for ROS production in mammalian cells (Tarafdar and Pula, 2018). NADPH is critical for the antioxidant system and reductive biosynthesis, and is required by enzymes involved in the pathologies of metabolic disorders due excessive ROS generation (Gomez-Sanchez *et al*., 2008; Legeza *et al*., 2013). Therefore, we determined the NADP/NADPH ratio, and the results showed that $PM_{2.5}$ increased the oxidized NADP ratio, while NIA decreased the this NADP ratio. In addition, NIA cleared intracellular ROS induced by $PM_{2.5}$.

Next, we examined the effect of $PM_{2.5}$ on the three main molecules in cells, namely lipids, proteins, and DNA. ROS is known to damage lipids and proteins via lipid peroxidation and protein carbonylation, respectively (Hyun *et al*., 2019a), as well as damaging DNA stability. Lipid peroxidation is regarded as a general oxidative process, and is related to cell death. Furthermore, ROS accumulation, induced by stimuli such as tobacco smoke, can directly damage lipids (Ayala *et al*., 2014). Our results showed that $PM_{2.5}$ exposure damaged lipids, while NIA protected the lipids from peroxidation caused by $PM_{2.5}$. Oxidative stress also increased protein carbonylation, which is associated with cell damage and cell disease and is considered one of the most harmful irreversible protein modifications (Fedorova *et al*., 2014). NIA showed preventive action against protein oxidation induced by $PM_{2.5}$. Previous study noted that $PM_{2.5}$ caused early arrest of cell cycle, resulting from induced DNA damage, and 8-oxoG, the oxidative stress biomarker, is the predominant adduct of ROS-induced oxidative changes (Abbas *et al*., 2019). NIA has been shown to improve DNA repair following damage induced by ultraviolet radiation (Park *et al*., 2010; Snaidr *et al*., 2019). In our study, we evaluated three indices of DNA stability (8-OHdG levels, 8-oxoG intensity, and DNA tails), and all results showed that NIA protected cells from $PM_{2.5}$-induced DNA lesions. Thus, NIA protects cells from $PM_{2.5}$-induced damage.

Calcium plays a key role in cell survival, as well as it can improve apoptosis. Ca^{2+} mediated pro-apoptotic action is a response to many endogenous organelles, including mitochondria (Hajnoczky *et al*., 2003). Ca^{2+} from endoplasmic reticulum plays an important role in mitochondria-related metabolic activity (Sarasija *et al*., 2018). The increased intracellular Ca^{2+} affects $\Delta\psi_m$ and the disruption of mitochondrial membrane permeability is related to cell death (Kroemer *et al*., 2007; Zorova *et al*., 2018). During the process of apoptosis, mitochondria receive pro-apoptotic signals and release pro-apoptotic proteins (Orrenius *et al*., 2015). Notably, $PM_{2.5}$ can induce cell apoptosis via various pathways, such as disruption of mitochondrial polarization, DNA fragmentation, and expression of pro-apoptotic proteins (Peixoto *et al*., 2017). All our results demonstrated that $PM_{2.5}$ increased intracellular Ca^{2+} levels, disrupted the balance of mitochondrial potential, and promoted apoptosis, NIA treatment could inhibit these effects.

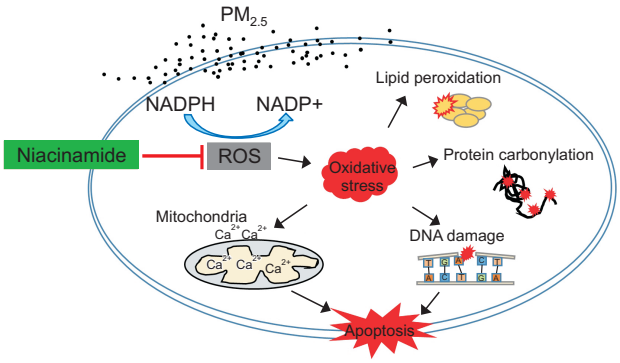

Fig. 7. Schematic diagram showing the protective action of NIA on $PM_{2.5}$-induced cell damage. NIA protected keratinocytes by suppressing ROS generation by decreasing the NADP/NADPH ratio. Further, NIA prevented oxidative stress-induced molecules damage, including lipid peroxidation, protein carbonylation, and DNA modification. NIA could also stabilize mitochondrial membrane potential by balancing calcium levels, which was disrupted by $PM_{2.5}$. Finally, NIA protected cells from $PM_{2.5}$-induced apoptosis.

Taken together, our findings show that $PM_{2.5}$ notably aggravated skin cell damage by inducing ROS generation, disrupting cellular components, and activating apoptotic pathways. However, cells pre-treated with NIA were protected from the ROS-induced lipid peroxidation, protein carbonylation, and DNA damage (Fig. 7). NIA also inhibited $PM_{2.5}$-induced apoptosis by maintaining both Ca^{2+} levels and mitochondrial membrane potential in a steady state. These results suggest that NIA can protect against $PM_{2.5}$-induced skin damage.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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