Niacinamide Protects Skin Cells from Oxidative Stress Induced by Particulate Matter

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Abstract
Niacinamide (NIA), also known as nicotinamide, 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 (PM₂.₅) 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 PM₂.₅-induced oxidative stress in human HaCaT keratinocytes. We found that NIA could inhibit the ROS generation induced by PM₂.₅, as well block the PM₂.₅-induced oxidation of molecules, such as lipids, proteins, and DNA. Furthermore, NIA alleviated PM₂.₅-induced accumulation of cellular Ca²⁺, which caused cell membrane depolarization and apoptosis, and reduced the number of apoptotic cells. Collectively, the findings show that NIA can protect keratinocytes from PM₂.₅-induced oxidative stress and cell damage.

Key Words: Niacinamide, Particulate matter 2.5, Oxidative stress, Human HaCaT keratinocyte
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-Isoprostanate 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-acetoxyethyl 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′-tetraethylbenzimidazol-carboxyanine iodide (DiBAC$_4$(3), Molecular Probes, Portland, OR, USA) according to the manufacturer’s instructions (Park et al., 2011).
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 PM₂.₅ 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 PM₂.₅ at a concentration of 50 μg/mL for ROS induction. This concentration was selected based on the findings of our recent study investigating whether PM₂.₅ induced oxidative stress. In that study, we measured ROS generation at various concentrations (25-100 μg/mL) of PM₂.₅, and 50 μg/mL PM₂.₅ was found to be optimal concentration

**Fig. 2.** NIA cleared ROS by inhibiting NOX activity induced by PM₂.₅. (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 PM₂.₅. *p<0.05 and #p<0.05 compared with control cells and PM₂.₅-exposed cells, respectively.

**Fig. 3.** NIA protected lipids from PM₂.₅-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 PM₂.₅-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 PM2.5 (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 PM2.5 increased the oxidation of NADPH, which was reversed by NIA pre-treatment (Fig. 2A). In the DCF-DA staining assay, NIA inhibited PM2.5-induced fluorescence in the cells, demonstrating that NIA protected cells from PM2.5-induced ROS generation (Fig. 2B). Similarly, DHE staining revealed that superoxide generation induced by PM2.5 was blocked by NIA (Fig. 2C). These results further indicated that NIA exerted antioxidant effects in keratinocytes.

**NIA inhibited PM2.5-induced lipid peroxidation**

The detection of phosphine oxide (DPPP oxide) using the DPPP probe indicated that PM2.5 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 PM2.5-induced peroxidation (Fig. 3B). These results showed that NIA rescued cells from PM2.5-induced lipid oxidation.

**NIA suppressed PM2.5-induced oxidative stress on proteins**

The levels of protein carbonylation, a specific indicator of protein oxidation, revealed that NIA significantly inhibited PM2.5-induced oxidative protein modification (Fig. 4). These results showed that NIA rescued cells from PM2.5-induced protein carbonylation.

**NIA protected cells from PM2.5-induced DNA damage**

The 8-OHdG assay was used to assess oxidative damage on DNA caused by PM2.5 (Fig. 5A). Notably, NIA reduced 8-OHdG generation induced by PM2.5. Furthermore, confocal microscopy showed that the level of 8-oxoG in PM2.5-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 PM2.5-induced oxidative stress. Protein oxidation was evaluated by protein carbonylation level. *p<0.05 and #p<0.05 compared with control cells and PM2.5-exposed cells, respectively.](image)

![Fig. 5. NIA protected DNA from PM2.5-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 PM2.5-induced DNA damage. *p<0.05 and #p<0.05 compared with control cells and PM2.5-exposed cells, respectively.](image)
of NIA on DNA damage was also observed in the comet assay. Length of comet tails and the percentage of tail fluorescence were increased by PM2.5, but decreased following NIA pretreatment (Fig. 5C). Collectively, these results illustrated that NIA protected against DNA damage induced by PM2.5.

**NIA lowered excessive intracellular Ca^{2+}, balanced membrane potential, and suppressed apoptosis induced by PM2.5**

Intracellular Ca^{2+}, tracked by Fluo-4-AM, was detected by confocal microscopy. The image analysis revealed that PM2.5 stimulated excessive Ca^{2+}, which could be reduced by NIA treatment (Fig. 6A). JC-1 staining was used to determine Δψ_{m}, with red and green fluorescence representing polarization and depolarization, respectively. The images obtained from confocal microscopy showed that Δψ_{m} polarization and depolarization were decreased and increased by PM2.5 treatment, respectively; however, they were reversed by NIA pretreatment (Fig. 6B). PM2.5 also promoted apoptotic bodies, when observed using Hoechst 33342 staining, and NIA pretreatment reduced their numbers (Fig. 6C). These results proved that PM2.5 disrupted the homeostasis of intracellular Ca^{2+} levels and accelerated cell apoptosis, but NIA exerted cytoprotective effects against these PM2.5-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...
Our results showed that PM2.5 exposure damaged lipids, while tobacco smoke can directly damage lipids (Ayala 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 PM2.5-induced oxidative damage in keratinocytes. NIA pretreatment at all tested concentrations inhibited PM2.5-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 (Kamprath et al., 2011; Sticocuzzi et al., 2012). NOXs are widely distributed in mammalian tissues and, thereby, serve various functions (Touyz et al., 2019). The six homologs of the cytoskeleton 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 PM2.5 increased the oxidized NADPH ratio, while NIA decreased the this NADPH ratio. In addition, NIA cleared intracellular ROS induced by PM2.5.

Next, we examined the effect of PM2.5 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 PM2.5 exposure damaged lipids, while NIA protected the lipids from peroxidation caused by PM2.5. 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 PM2.5. Previous study noted that PM2.5 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; Snaird 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 PM2.5-induced DNA lesions. Thus, NIA protects cells from PM2.5-induced damage.

Calcium plays a key role in cell survival, as well as it can improve apoptosis. Ca2+ mediated pro-apoptotic action is a response to many endogenous organelles, including mitochondria (Hajnoczky et al., 2003). Ca2+ from endoplasmic reticulum plays an important role in mitochondria-related metabolic activity (Sarasija et al., 2018). The increased intracellular Ca2+ affects Δψm 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, PM2.5 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 PM2.5 increased intracellular Ca2+ levels, disrupted the balance of mitochondrial potential, and promoted apoptosis, NIA treatment could inhibit these effects. Taken together, our findings show that PM2.5 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 PM2.5-induced apoptosis by maintaining both Ca2+ levels and mitochondrial membrane potential in a steady state. These results suggest that NIA can protect against PM2.5-induced skin damage.

**CONFLICT OF INTEREST**

The authors declare that there are no conflicts of interest.

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