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Chemoprotective effect of N-acetylcysteine (NAC) on cellular oxidative damages and apoptosis induced by nano titanium dioxide under UVA irradiation

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ABSTRACT

The chemoprotective effect of N-acetylcysteine (NAC), a sulfhydryl-containing antioxidant, on nano titanium dioxide (nano-TiO_2) induced oxidative stress and apoptosis in human keratinocyte (HaCaT) cells was assessed. HaCaT cells were pretreated with NAC followed by treatment with 200 μg/ml nano-TiO_2, then exposed to ultraviolet A (UVA, 365 nm) for 1 h and cultured for 24 h. Intracellular reactive oxygen species (ROS) and nitric oxide (NO) formation, mitochondrial membrane potential (MMP), apoptosis and the content of the lipid peroxidation product malondialdehyde (MDA) were measured. Keratin 6 (K6) mRNA expression was also analyzed. The results showed that NAC strongly inhibited ROS and NO production in nano-TiO_2 treated cells. The extent of lipid peroxidation was also decreased in the presence of NAC. In addition, NAC suppressed nano-TiO_2 induced apoptosis and increased K6 mRNA expression. The results indicated that NAC could prevent oxidative stress and apoptosis induced by nano-TiO_2 in HaCaT cells.

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1. Introduction

Nano titanium dioxide (nano-TiO_2) has been widely used in the production of plastic, paper, paint, welding rod-coating material, and food colorant (Gurr et al., 2005). Without ultraviolet (UV) irradiation, nano-TiO_2 was shown to have no inflammatory effect or genotoxicity in the rat (Rehn et al., 2003), and induced no DNA damages in human cells (Dunford et al., 1997; Nakagawa et al., 1997). In contrast, several studies have reported that nano-TiO_2 caused inflammation (Park et al., 2008), and increased in micronuclei induction and apoptosis (Rahman et al., 2002). Nano-TiO_2 was also reported to affect gene expressions, including an apoptosis-related gene (Carinci et al., 2003). However, nano-TiO_2 is known to be a photobiologically active agent (Gurr et al., 2005). When exposed to UV irradiation, nano-TiO_2 was considered to drive the generation of ROS, such as superoxide anion radicals, hydrogen peroxide, free hydroxyl radicals, and singlet oxygen (Hirakawa et al., 2004). ROS generated following UV irradiation of nano-TiO_2 are genotoxic in Chinese hamster and mouse lymphoma cell lines (Nakagawa et al., 1997), and are cytotoxic to cultured HeLa cells (Cai et al., 1992), human fibroblasts (Wamer et al., 1997), Chinese hamster ovary cells (Uchino et al., 2002), and human colon carcinoma Ls-174-t cells (Zhang and Sun, 2004). Owing to the existence of nano-TiO_2 in environment and sunscreen products, skin is inevitable to become potential target site. However, little information is available concerning the skin toxicities and irritation potentials of nano-TiO_2. In our previous work, UVA lamp (365 nm, 3.5 mW/cm^2) was used as a substitute for UVA occurring in natural sunlight and found that nano-TiO_2 could induce ROS generation and oxidative damage in human keratinocyte (HaCaT) cells. And P25 (anatase–rutile mixture, consists of 75% anatase and 25% rutile), which is the most widely used nano-TiO_2 in the world, showed higher oxidative damage effect than that of anatase and rutile form (Xue et al., 2010). Topical application of nano-TiO_2 for a prolonged period can induce dermal toxicity, most likely associated with free radical generation, oxidative stress, and collagen depletion that can lead to skin aging (Wu et al., 2009).

The N-acetylcysteine (NAC) as an antioxidant and free radical scavenger is used extensively in conditional nutrient (Moschou et al., 2008). NAC acts as a cysteine donor and maintains or even increases the intracellular levels of glutathione, a tripeptide which protects cells from toxins such as free radicals. Reports have shown the ability of antioxidants such as NAC to reduce cell damage induced by cadmium (Smith et al., 2009), or dental composite (Stanislawski et al., 2000, 2003).

In this study, we investigated the chemoprotective effects of NAC on nano-TiO_2 (P25) induced oxidative stress and apoptosis under UVA irradiation and showed the first evident of the inhibition of nano-TiO_2 induced HaCaT cells damage by NAC. The actual

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mechanisms of NAC are also elucidated. These include the inhibition of nano-TiO_2 induced intracellular ROS production, lipid peroxidation, and apoptosis. The research work may give some advices for expanding application fields of nano-TiO_2.

2. Materials and methods

2.1. Chemicals

Nano-TiO_2 (P25, 25% rutile and 75% anatase) with 21 nm was purchased from Degussa GmbH (Germany). HaCaT cells were purchased from China Center for Type Culture Collection (CCTCC, China), Rhodamine (Rh) 123, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium-bromide (MTT) were purchased from Sigma (USA). Modified Eagle's medium (MEM), penicillin–streptomycin and fetal bovine serum (FBS) were obtained from Hyclone laboratories Inc (USA). All other chemicals were of the highest grade that could be obtained commercially.

2.2. Preparation suspension of nano-TiO_2

Nano-TiO_2 was suspended in the cell culture medium and dispersed using a sonifier cell disruptor equipped with a microprobe for 10–15 min to produce suspension. In each study, the suspension was freshly prepared and then immediately applied to HaCaT cells.

2.3. Cell treatment

HaCaT cells were grown in modified Eagle's medium (MEM) supplemented with 10% FBS in the presence of 5% CO_2 in air at 37 °C. Cells were incubated up to about 24 h and grown to about 80% confluence before experiments. NAC (Sigma–Aldrich, USA) was dissolved in PBS (500 mM) and added to culture medium reaching a concentration of 5 mM and pretreated for 2 h, after treatment, NAC was removed and cells were washed with phosphate buffered saline (PBS) and replaced with culture medium, some experiments included treatment with nano-TiO_2. All cells were irradiated with the UVA light for 1 h and then cultured for 24 h.

2.4. UVA irradiation

UVA light was provided by a UV lamp (Shanghai Jinguang Instrument, China) with continuous emison spectrum with a peak at 365 nm. The UV intensity at 365 nm was determined using a UVA radiometer (Beijing Normal University, China). The UVA lamp had a fluence rate of 3.5 mW/cm^2, and is an appropriate substitute for UVA occurring in natural sunlight.

2.5. Cell viability assay

The cytotoxicity of particles was determined using the MTT assay as described (Mosmann, 1983; Young et al., 2005). Briefly, cells were plated at a density of 1000 cells/well in a 96 well culture plate. After treatment, cells were washed with phosphate buffered saline (PBS) and incubated at 37 °C with 5 mg/ml MTT (M2003, Sigma–Aldrich, USA) for 4 h. The conversion of the tetrazolium salt MTT to a colored formazan by mitochondrial dehydrogenase was used to assess cell viability. After the supernatant was removed, 150 μl of DMSO was added to each well and the absorbance was read at 490 nm.

2.6. Flow cytometric analysis of cell apoptosis

Apoptotic cells were quantified using an Annexin V-FITC apoptosis detection kit (A9210, Sigma–Aldrich, USA). After treatment, Cells were washed twice in cold PBS and then treated with trypsin–EDTA for 5 min. Cells were centrifuged at 1000 rpm for 5 min and the pellets were resuspended in binding buffer at a density of 1×10^6 cells/ml. 100 μl of the solution was transferred to a culture tube and double-stained with 5 μl of Annexin V-FITC and 10 μl of PI (Sigma, USA), and the cells were incubated at room temperature for 15 min in the dark. Cell suspensions were readjusted to a final volume of 500 μl with 1× Annexin V binding buffer for further analysis by flow cytometry (FACScan, Becton Dickinson LSR, USA).

2.7. Lactate dehydrogenase (LDH) release assay

Cytotoxicity induced by nano-TiO_2 was also assessed by LDH leakage into the culture medium. Following exposure to nano-TiO_2, the cells were harvested and the LDH activity was assayed spectrophotometrically following the decrease in the absorbance of NADH at 340 nm by LDH assay kit (Jiancheng Ltd., China).

2.8. Intracellular ROS formation

The generation of ROS was determined using a fluorescein-labeled dye, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) (Xiong et al., 2006).This nonfluorescent dye permeated cells easily and was hydrolyzed to fluorescent 2′,7′-dichlorofluorescein (DCF) upon interaction with intracellular ROS. Cells were plated at a density of 10,000 cells/well in a 24 well culture plate. After treatment, cells were washed with PBS and incubated with 10 μM DCFH-DA for 30 min at 37 °C. Some of the stained cells were immediately visualized under a fluorescent microscope. Others were washed with PBS and then homogenized in 300 μl of 0.1% Triton X-100 (PBS, pH 7.4) through sonication on ice for 10 s. After incubation at 4 °C for 10 min, the homogenates were centrifuged at 12,000 rpm for 30 min. The supernatants were used for assay with excitation wavelength at 488 nm and emission wavelength at 525 nm by fluorospectrophotometer.

2.9. Measurement of NO

The amount of stable nitrite (nitrite and nitrate), the end product of NO released from HaCaT cells in culture was quantified colorimetrically by measuring the accumulation of nitrite in culture medium (Angelo et al., 2001). Briefly, 100 μl of culture supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrocholoride, 2.5% H_3PO_4), and incubated at room temperature for 10 min. The absorbance was read at 540 nm using microplate reader. Nitrite concentration was determined by comparison with a standard of $NaNO_2$.

2.10. Measurement of intracellular malondialdehyde (MDA)

The thiobarbituric acid assay (TBARS) was used to detect lipid peroxidation (Draper and Hadley, 1990). After treatment, cells were washed with PBS and homogenized in 300 μl 0.1% Triton X-100 (PBS, pH 7.4) through sonication on ice for 10 s. After incubation at 4 °C for 10 min, the homogenates were centrifuged at 10,000 rpm for 10 min, and the supernatants were used for assay. MDA content was measured with the MDA kit (Jiancheng Ltd., China).

2.11. Evaluation of mitochondrial membrane potential (MMP) by flow cytometry

MMP of the cells was monitored using a flow cytometer and the fluorescent dye Rhodamine (Rh) 123, a cell permeable cationic dye which preferentially enters into mitochondria based on the highly negative MMP. Depolarization of MMP results in the loss of Rh 123

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