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Regulation of Plasminogen Activator Inhibitor-1 Expression in Endothelial Cells with Exposure to Metal Nanoparticles

Min Yu^{*,1,3}, Yiqun Mo^{*,1}, Rong Wan^{*,1}, Sufan Chien², Xing Zhang³, and Qunwei Zhang¹

¹Department of Environmental and Occupational Health Sciences, School of Public Health and Information Sciences, University of Louisville, Louisville, KY, USA

²Department of Surgery, School of Medicine, University of Louisville

³Department of Hygiene, Zhejiang Academy of Medical Sciences, Zhejiang, P.R. China

Abstract

Recent studies demonstrated that exposure to nanoparticles could enhance the adhesion of endothelial cells and modify the membrane structure of vascular endothelium. The endothelium plays an important role in the regulation of fibrinolysis, and imbalance of the fibrinolysis system potential contributes to the development of thrombosis. Plasminogen activator inhibitor-1 (PAI-1) is the most potent endogenous inhibitor of fibrinolysis and is involved in the pathogenesis of several cardiovascular diseases. The aim of this study was to investigate the alteration of PAI-1 expression in mouse pulmonary microvascular endothelial cells (MPMVEC) exposed to the metal nanoparticles that are known to be reactive, and the potential underlying mechanisms. We compared the alteration of PAI-1 expression in MPMVEC exposed to non-toxic doses of nano-size copper (II) oxide (Nano-CuO) and nano-size titanium dioxide (Nano-TiO₂). Our results showed that Nano-CuO caused a dose- and time-dependent increase in PAI-1 expression. Moreover, exposure of MPMVEC to Nano-CuO caused reactive oxygen species (ROS) generation that was abolished by pretreatment of cells with ROS scavengers or inhibitors, DPI, NAC and catalase. Exposure of MPMVEC to Nano-CuO also caused a dose- and time-dependent increase in p38 phosphorylation by Western blot. These effects were significantly attenuated when MPMVEC were pre-treated with DPI, NAC and catalase. To further investigate the role of p38 phosphorylation in Nano-CuO-induced PAI-1 overexpression, the p38 inhibitor, SB203580, was used to pre-treat cells prior to Nano-CuO exposure. We found that Nano-CuO-induced overexpression of PAI-1 was attenuated by p38 inhibitor pretreatment. However, Nano-TiO₂ did not show the same results. Our results suggest that Nano-CuO caused upregulation of PAI-1 in endothelial cells which is mediated by p38 phosphorylation due to oxidative stress. These findings have important implications for understanding the potential health effects of metal nanoparticle exposure.

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Address Correspondence To: Qunwei Zhang, MD, MPH, PhD, Department of Environmental and Occupational Health Sciences, School of Public Health and Information Sciences, University of Louisville, 485 E. Gray Street, Louisville, KY 40209, Tel: (502)852-7200, Fax: (502)852-7246, Qunwei.Zhang@louisville.edu or Xing Zhang, MD, PhD, Department of Hygiene, Zhejiang Academy of Medical Sciences, 182 Tian Mu Shan Road, Hangzhou, Zhejiang, 310013, P.R. China, Tel: +86-571-88215459, Fax: +86-571-88075447, xingtyou@mail.hz.zj.cn.

*These authors contributed equally to this work.

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Keywords

Metal nanoparticles; Plasminogen activator inhibitor-1; p38 phosphorylation; Reactive oxygen species

1. Introduction

Nanoparticles are now widely applied in the cosmetics, electronics and medical fields. Some metal nanoparticles, such as nano-size copper oxide (Nano-CuO), nano-size nickel (Nano-Ni) and nano-size cobalt (Nano-Co), with a high level of surface energy, high magnetism, low melting point, and high surface area, have been widely used in industry, e.g. in magnetic tape, an additive in lubricants, metallic coating and as a highly reactive catalyst in organic hydrogen reactions. Because of their unique physicochemical properties, nanoparticles may pose potential hazards for human health (Kubik et al., 2005; Maynard et al., 2006). Metal nanoparticles, in particular, have been shown to cause significant biological effects. For example, exposure to Nano-CuO, Nano-Ni or Nano-Co caused greater oxidative stress and inflammatory response compared to larger sized particles at the equivalent mass concentration (Chen et al., 2006; Karlsson et al., 2008, 2009; Meng et al., 2007; Zhang et al., 2000, 2003). These studies suggest that some metal nanoparticles are substantially more toxic than larger sized metal particles (Chen et al., 2006; Karlsson et al., 2008, 2009; Meng et al., 2007; Zhang et al., 2000, 2003). The current studies on the biological effects of Nano-TiO₂ are controversial because Nano-TiO₂ is composed of rutile, anatase and brookite, and different formulations of Nano-TiO₂ may cause different biological effects (Johnston et al., 2009; Oberdörster et al., 2005).

Previous studies have shown that nanoparticles can penetrate rapidly through the epithelium to the endothelium (Nemmar et al., 2001, 2002b; Shimada et al., 2006). They may even enter the circulatory system and translocate to extra-pulmonary tissues, which may cause potential health effects on extra-pulmonary organs (Nemmar et al., 2001, 2002a, b; Oberdörster et al., 2002a, b, 2005; Takenaka et al., 2001). Recent studies also demonstrated that exposure to nanoparticles could enhance adhesion of endothelial cells, and modify the membrane structure of vascular endothelium (Frampton et al., 2006). The interactions between pulmonary microvascular endothelial cells and nanoparticles are important because pulmonary microvascular cells are the first target cells when the nanoparticles are translocated from the lungs to the circulatory system. Exposure to certain metal nanoparticles also induces microtubule remodeling, increasing endothelial cell permeability (Apopa et al., 2009). The presence of nanoparticles in the circulation could also influence cardiovascular function; previous studies have shown that exposure to ambient ultrafine particles may activate endothelial cells which may further cause systemic inflammation and dysfunction of the endothelium (Mo et al., 2009). Nano-CuO are widely used in antimicrobial preparations, polymers/plastics, metallic coating and ink, semiconductors or intrauterine contraceptive devices (Chen et al., 2006; Aruoja et al., 2009). However, few studies have investigated the direct effects of Nano-CuO on endothelial cells and the potential mechanisms involved in these effects (Aruoja et al., 2009; Karlsson et al., 2008, 2009; Meng et al., 2007).

Plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor family, plays a major role in inhibition of plasminogen activators; PAI-1 is implicated in the pathogenesis of myocardial infarction and stroke (Johansson et al., 2000; Scarabin et al., 1998). PAI-1 is synthesized in the liver, endothelial cells, vascular smooth muscle cells and macrophages (Chen et al., 2008). The level of PAI-1 in endothelial cells is related to the dynamic status of coagulation, and PAI-1 expression is highly regulated by many factors

including cytokines, oxidative stress, and cellular signaling molecule such as mitogen-activated protein kinases (MAPKs) (Jaulmes et al., 2009; Oszejka et al., 2008; Swiatkowska et al., 2002; Zhao et al., 2009). Elevated PAI-1 is closely associated with enhanced thrombosis by impairing fibrinolysis (Dawson et al., 1992; Hamsten et al., 2000), which has been recognized as an important risk factor of atherosclerotic vascular disease (Hamsten et al., 2000). Exposure to ambient particulate matter (PM) and ultrafine carbon has been shown to increase the PAI-1 concentration in plasma (Cozzi et al., 2007; Upadhyay et al., 2008).

In the present study, we first investigated PAI-1 regulation in endothelial cells exposed to Nano-CuO and Nano-TiO₂, and then studied the potential mechanisms and signaling pathways that may be involved in these effects. We propose that exposure of endothelial cells to non-toxic doses of Nano-CuO causes ROS generation, which will activate the phospho-p38 pathway, impairing endothelial fibrinolytic activity through up-regulation of PAI-1 expression.

2. Materials and Methods

2.1. Metal nanoparticles and their characterization

Nano-size copper (II) oxide (Nano-CuO) with a diameter of 42 nm was purchased from Sigma-Aldrich (St. Louis, MO). Nano-TiO₂ with a mean diameter of 28 nm was provided by INABTA and Co., Ltd., Vacuum Metallurgical Co., Ltd., Japan. The microstructure of Nano-CuO and Nano-TiO₂ was characterized by transmission electron microscopy (TEM) (Karlsson et al., 2008; Zhang et al., 1998). Nano-CuO or Nano-TiO₂ was dispersed in physiological saline and ultrasonicated for about 30 min prior to each experiment. The characteristics of these nanoparticles have been described and summarized in previous studies (Karlsson et al., 2008; Mo et al., 2008; Zhang et al., 1998). Briefly, the specific surface area is 23 m²/g for Nano-CuO and 45 m²/g for Nano-TiO₂. The size of particles and agglomerates in cell medium is 220 nm for Nano-CuO and 280 nm for Nano-TiO₂ which were measured by dynamic light scattering (DLS) (Karlsson et al., 2008; Mo et al., 2008) and is summarized in Table 1.

2.2. Chemicals and reagents

2', 7'-dichlorodihydrofluorescein diacetate (H₂-DCFDA) was obtained from Molecular Probes (Eugene, OR), diphenyleneiodonium chloride (DPI) from ALEXIS (San Diego, CA), catalase (CAT) and N-acetyl-L(+)-cysteine (NAC) from Fisher (Fair Lawn, NJ), and SB203580 from Tocris bioscience (Ellisville, MO). Monoclonal mouse anti- β -actin antibody was purchased from Sigma (Saint Louis, MO), antibodies against total p38 and phospho-p38 from Cell Signaling Technology (Beverly, MA), antibody against PAI-1 and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG from Santa Cruz Biotechnology (Santa Cruz, CA), HRP-conjugated goat anti-rabbit IgG from CHEMICON (Temecula, CA). The ECL Western Blotting Detection Reagents was obtained from GE Healthcare, Amersham™ (Buckinghamshire, UK). Dulbecco's Modification of Eagle's Medium (DMEM), fetal bovine serum (FBS), 0.05% trypsin EDTA, nonessential amino acids, and penicillin–streptomycin solution were purchased from Mediatech, Inc. (Manassas, VA). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ) except when otherwise stated. All chemicals used were of analytical grade.

2.3. Endothelial cell culture

Mouse pulmonary microvascular endothelial cells (MPMVEC) were isolated from C57BL/6J mice as previously described (Zhang et al., 2008; Mo et al., 2009). MPMVEC were cultured in a 5% CO₂ atmosphere at 37°C in DMEM with 4.5g/l glucose, L-glutamine and

sodium pyruvate, supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, and 1% nonessential amino acids.

2.4. Cytotoxicity of metal nanoparticles

The cytotoxicity of Nano-CuO or Nano-TiO₂ in MPMVEC was determined by both AlamarBlue™ assay (AbD Serotec, Oxford, UK) and CellTiter 96 AQueous non-radioactive cell proliferation assay (MTS assay) (Promega, Madison, WI) according to the manufacturer's instruction. **AlamarBlue™ assay** is a colorimetric/fluorometric method for determining the number of metabolically active cells through oxidation-reduction indicator. Briefly, 2×10^3 cells per well were seeded into 96-well plates and were allowed to attach to the growth surface by incubating overnight. MPMVEC were then treated with different concentrations (0, 0.625, 1.25, 2.5, 5, 7.5 and 10 µg/ml) of Nano-CuO or Nano-TiO₂ **in a total volume of 200 µl per well** for 24h. The fluorescence absorbance at 570 and 600 nm was recorded with a multi-detection microplate reader (Synergy HT, BioTek, Winooski, VT). The cell viability was expressed as the percentage of the control which was without treatment. Another method, MTS assay, is a colorimetric method for determining the number of metabolically active cells in which the dehydrogenase can convert a tetrazolium compound (MTS) into an aqueous, soluble, and colored formazan. This method was performed as in our previous study (Mo et al., 2009).

2.5. Endothelial cell ROS generation

Dichlorofluorescein (DCF) fluorescence was used to monitor ROS generation with exposure to metal nanoparticles as previously described (Mo et al., 2009 a, b; Wan et al., 2008). The nonfluorescent cell-permeant form, 2', 7'-dichlorodihydrofluorescein diacetate (H₂-DCFDA), rapidly diffuses through the cell membrane and is hydrolyzed by intracellular esterases to an oxidative sensitive form, dichlorodihydrofluorescein (H₂-DCF). This serves as a substrate for intracellular oxidants to generate highly fluorescent DCF, with a fluorescent intensity proportional to intracellular ROS. A 20 mM stock solution of H₂-DCFDA was prepared in 100% ethanol, stored at -20°C in the dark. After cells were seeded into a 96-well plate and allowed to attach to the growth surface by culturing overnight, cells were incubated for 2 h with H₂-DCFDA at 37°C in the dark by adding it directly to the cell culture medium to make a final concentration of 5 µM. Then cells were treated with 0, 0.625, 1.25 and 2.5 µg/ml of Nano-CuO or Nano-TiO₂ for 12h. Cells without metal nanoparticle exposure were used as control. The fluorescence of DCF was measured using Synergy HT microreader (BioTek, Winooski, VT) at 485 nm excitation (λ_{ex}) and 528 nm emission (λ_{em}). Cell fluorescence without the addition of H₂-DCFDA was used as background fluorescence level and subtracted out from the sample fluorescence level. Results, in arbitrary fluorescence units (AFU), were expressed according to the ratio [(AFU in treated cells) / (AFU in control cells)] × 100.

To examine the role of antioxidants on ROS generation in MPMVEC after exposure to Nano-CuO, MPMVEC were pretreated with ROS scavengers or inhibitors such as NAC (20 mM), DPI (10 µM), or CAT (1000 U/ml) for 2 h, then cells were preloaded with 5 µM H₂-DCFDA for another 2 h prior to exposure to 2.5 µg/ml of Nano-CuO for 12 h. Fluorescence values were measured as described above.

2.6. Protein extraction and Western blot

Total MPMVEC lysates were extracted with 2×SDS gel sample buffer (20 mM dithiothreitol, 6% SDS, 0.25 M Tris, pH 6.8, 10% glycerol, 10 mM NaF and bromophenyl blue) at 1×10^6 cells per 100 µl. If only cytosolic proteins were needed for Western blot, the cytosolic proteins were extracted by using RIPA lysis buffer (Santa Cruz, CA) supplemented with PMSF, protease inhibitor cocktail and sodium orthovanadate (Santa Cruz). Cells were

lysed in RIPA or 2×SDS gel sample buffer on ice for 40 min. The supernatant was collected after centrifuging at 10,000 ×g and 4 °C for 15 min. The protein concentration in the supernatant was measured by Protein Assay (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard or was expressed by counting cell number.

Western blot was performed as previously described with minor modifications (Mo et al., 2009; Zhang et al., 2008). Briefly, 40 µg protein (for phospho-p38 and total p38) or proteins extracted from 4×10⁵ cells (for PAI-1) were loaded in each lane of 12% polyacrylamide gel in the presence of 0.1% SDS. Electrophoresis was carried out in a 0.025 M Tris/0.192 M glycine/0.1% SDS buffer, pH 8.3. EZ-Run™ pre-stained protein marker (Fisher, Pittsburgh, PA) was used to check the molecular weight of target proteins. After electrophoresis, proteins were electrotransferred into Immun-Blot polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA). The blot was blocked by incubation in 5% milk in 1 × TBST (10 mM Tris/150 mM NaCl/0.05% Tween-20, pH 7.5) for 2 h at room temperature or overnight at 4 °C, and incubated with primary antibodies at 4 °C overnight with gentle shaking. The blot was washed with 1 × TBST three times, incubated with HRP-conjugated secondary antibodies for 1 h, then exhaustively washed with 1 × TBST three times. Immunoreactive bands were detected by chemifluorescence using ECL Western Blotting Detection Reagents (Amersham Biosciences) following developing film (Amersham Biosciences) and quantified by using Gel Doc XR (Bio-Rad, Hercules, CA).

2.7. RNA isolation and semi-quantitative RT-PCR

Total RNA was extracted from MPMVEC using TRIZOL Reagent (Sigma, St. Louis, MO) according to the manufacturer's instruction. Two microgram of total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, WI) in a total volume of 25 µl. A total of 1 µl of cDNA, 1 µl of 5 µM each primer, 0.5 µl of 10 mM dNTP, 1.5 µl MgCl₂ (25 mM), 5 µl 5× Green GoTaq Flexi Buffer and 0.25 µl GoTaq Flexi DNA polymerase (5 U/µl) (Promega, Madison, WI) were used in each PCR reaction at a final volume of 25 µl. The PCR reaction was performed on a Mastercycler (Eppendorf, Westbury, NY) using 30 cycles (for PAI-1) or 26 cycles (for β-actin) at 94°C for 45 s, at 58°C for 45 s, and at 72°C for 45 s. The primers for mouse PAI-1 were: 5'-GGG GCC GTG GAA CAA GAA TGA GAT -3' and 5'-AGA TGT TGG TGA GGG CGG AGA GGT-3'; for mouse β-actin were: 5'-GGC ATT GTT ACC AAC TGG GAC-3' and 5'-ACC AGA GGC ATA CAG GGA CAG -3'. After separation on a 1% agarose gel, PCR products were visualized and analyzed by Gel Doc XR (Bio-Rad, Hercules, CA). Intensities of target gene products were then normalized by that of mouse β-actin to obtain the relative densities.

2.8. Statistical Analysis

Values were presented as mean ± SD. For dose-response studies, differences among groups were evaluated with two-way analysis of variance; if the F-value was significant, groups were then compared at each dose by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. A value of *p* < 0.05 was considered significant. Statistical analyses were carried out using Sigma Stat (Jandel Scientific, San Raphael, CA).

3. Results

3.1. Cytotoxic effects of Nano-CuO or Nano-TiO₂ on MPMVEC

MPMVEC were exposed to various concentrations, ranging from 0 to 10 µg/ml, of Nano-CuO or Nano-TiO₂, for 24 h. Cell viability was not affected by any indicated concentration of Nano-TiO₂ by using AlarmaBlue™ assay (Fig. 1A). In contrast, exposure to 5, 7.5 and 10 µg/ml of Nano-CuO caused a dose-response decrease in cell viability (Fig. 1A). MPMVEC exposure to 0.625, 1.25 and 2.5 µg/ml of Nano-CuO did not show any significant

change in cell viability (Fig. 1A). These results were further conformed by using MTS assay (Fig. 1B). In the following experiments, non-toxic doses were chosen to observe the effects of Nano-CuO on endothelial cells.

3.2. ROS generation in MPMVEC exposed to Nano-CuO, but not to Nano-TiO₂

Exposure of MPMVEC to Nano-CuO caused a dose-response increase in ROS generation reflected by an increase in DCF fluorescence (Fig. 2A). Compared with control, exposure to 2.5 µg/ml of Nano-CuO for 12 h stimulated almost two-fold ROS generation. ROS was also significantly increased in MPMVEC exposed to 0.625 and 1.25 µg/ml of Nano-CuO for 12 h (Fig. 2A). However, exposure of MPMVEC to the same concentrations of Nano-TiO₂ did not cause DCF fluorescence increase (Fig. 2A). Pre-treatment of cells with ROS inhibitors or scavengers, such as NAC, CAT or DPI, prior to exposure to Nano-CuO significantly attenuated ROS generation (Fig. 2B).

3.3. Role of Nano-CuO-induced ROS generation on p38 activation

To examine whether Nano-CuO-induced ROS generation could activate endothelial cells, the effect of Nano-CuO on p38 mitogen-activated protein kinase (MAPK) was studied by Western blot. There were dose- and time-response increases in the phosphorylation of p38 after MPMVEC were exposed to 0.625, 1.25 and 2.5 µg/ml of Nano-CuO for 3 h (Fig. 3) or exposed to 2.5 µg/ml of Nano-CuO for 1, 3, 6 and 12 h (Fig. 4). However, exposure MPMVEC with Nano-TiO₂ did not cause increase in the phosphorylation of p38 (Fig. 3). To further investigate the role of ROS generation on Nano-CuO-induced p38 activation, MPMVEC were pre-treated with ROS inhibitors or scavengers, DPI (10 µM), CAT (1000 U/ml) or NAC (20 mM), 2 h prior to 2.5 µg/ml of Nano-CuO treatment for another 3 h. The results showed that Nano-CuO-induced increase of phosphorylation of p38 was significantly attenuated when MPMVEC were pre-treated with DPI, CAT or NAC (Fig. 5). These results indicated that ROS generation in MPMVEC exposed to Nano-CuO is involved in the activation of p38.

3.4. Effects of Nano-CuO on PAI-1 expression

The effects of Nano-CuO and Nano-TiO₂ on PAI-1 expression in MPMVEC were investigated at the transcriptional level by semi-quantitative RT-PCR and protein level by Western-blot. The results showed that there was a dose-response increase on PAI-1 mRNA level after exposure to Nano-CuO from 0 to 2.5 µg/ml for 3 h (Fig. 6). Consistent with transcription regulation, there was a dose-response increase in the PAI-1 protein level in MPMVEC exposed to Nano-CuO for 6 h (Fig. 6). The results also showed a time-response increase on PAI-1 mRNA expression from 1 to 12h when MPMVEC were exposed to 2.5 µg/ml of Nano-CuO (Fig. 7). However, Nano-TiO₂ did not cause a time-response increase in PAI-1 mRNA level (Fig. 7).

To determine whether induction of PAI-1 by Nano-CuO was associated with Nano-CuO-induced ROS, MPMVC were pre-treated with ROS inhibitor or scavenger for 2 h, such as DPI, NAC or CAT, prior to exposure to 2.5 µg/ml of Nano-CuO for 3 h. The results showed that DPI, NAC or CAT pre-treatment significantly reduced Nano-CuO-caused PAI-1 up-regulation (Fig. 8).

3.5. Nano-CuO-induced PAI-1 expression through activation of p38

To investigate the potential pathway involved in the Nano-CuO-induced PAI-1 up-regulation, we used a phospho-p38 specific inhibitor, SB203580 (5 and 10 µM), to pre-treat MPMVEC for 4 h prior to 2.5 µg/ml Nano-CuO treatment for 3 h. The results showed that Nano-CuO-induced PAI-1 up-regulation were significantly reduced by 5 µM or 10 µM of

SB203580 treatment (Fig. 9), demonstrating that PAI-1 overexpression after Nano-CuO treatment is through activation of p38.

4. Discussion

Manufactured metal nanoparticles and their applications are continuously expanding due to their unique characteristics, such as small diameter, high level of surface energy, high magnetism, and high surface area. They are widely applied in industrial, cosmetics, and medicine. Thus, it becomes more and more important to understand the potential health effects of nanoparticles. There are three major points of contact for nanoparticles in the human body: the skin, the lung and the gastrointestinal tract (Baswas and Wu, 2005; Kubik et al., 2005; Maynard et al., 2006). Because of their small size, nanoparticles are not as readily phagocytized by macrophages as larger particles. They consequently can penetrate much more rapidly through the epithelium and reach the endothelium. They may even enter the blood circulation, resulting in their translocation to other organs (Nemmar et al., 2001, 2002a, b; Oberdörster et al., 2002a, b; Shimada et al., 2006). Translocation of particles also depends on the exposure route, dose, particle diameter, and surface chemical characteristics. Because endothelial cells are a possible target for metal nanoparticles when they translocate from the lungs to the circulatory system, we studied whether exposure to some metal nanoparticles, such as Nano-CuO or Nano-TiO₂ can activate endothelial cells and result in endothelial dysfunction. We also investigated the potential mechanism involved in these effects.

Our results showed a dose-response toxic effect on MPMVEC after exposure to Nano-CuO at concentrations ranging from 0 to 10 µg/ml. Exposure to Nano-CuO at concentrations 5 µg/ml and beyond caused significant cytotoxic effects. However, there were no toxic effects on MPMVEC with exposure to Nano-TiO₂ at all experimental doses. The mechanism of toxicity of nanoparticles is very complex. Several factors, such as small size, shape and chemical composition, may be involved in the toxic effects of nanoparticles. Previous studies showed that the toxicity of Nano-CuO was likely due to their small diameter and high surface area properties rather than Cu ions released to the cell medium (Karlsson et al., 2008). Although our *in vitro* studies can not predict the degree of human health effects at these concentrations, or the quantity of real exposure to nanoparticles that might be comparable to these dose-response studies, these dose-response studies provide a basis for organ toxicological studies. The use of doses that are lower than the cytotoxic dose can help identify potential toxic effects of nanoparticles not related to cytotoxicity.

Oxidative stress is a central hypothetical mechanism for the adverse effects of nanoparticles (Baswas and Wu, 2005; Oberdörster et al., 2005). Several studies have shown that nanoparticle-related excessive production of ROS is the leading effect driving adverse health effects (Baswas and Wu, 2005; Donaldson et al., 2003; Oberdörster et al., 2005; Zhang et al., 1998). Oxidative stress acts through redox-sensitive pathway, such as MAPK and NF-κB to result in inflammation (Donaldson et al., 2003). Though the components that mediate these effects differ among the different nanoparticles, there is commonality through their ability to cause oxidative stress and inflammation. In the present study, we used the fluorescence probe H₂DCF to determine ROS generation in MPMVEC after exposure to Nano-CuO. H₂DCF is non fluorescent when chemically reduced, but after cellular oxidation and removal of acetate groups by cellular esterases, it becomes fluorescent. Our results demonstrated a dose-response increase in DCF fluorescence in endothelial cells with exposure to Nano-CuO. Nano-CuO-induced ROS generation was blocked by pretreatment of endothelial cells with ROS inhibitors or scavengers, such as DPI, catalase, or NAC. These results suggest that exposure of endothelial cells to Nano-CuO caused ROS generation.

PAI-1 is a 50 kDa glycoprotein that acts as the primary physiological inhibitor of two main mammalian plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (Ha et al., 2009). PAI-1 can be synthesized by various cells, including hepatocytes, adipocytes, endothelial cells, and vascular smooth muscle cells, platelets, monocytes and macrophages. Among those cells, endothelial cells are one of the major sources of PAI-1. PAI-1 also plays a key role in the regulation of vascular and tissue remodeling (Fay, 2004; Gils & Declerck, 2004). The molecular mechanisms that regulate PAI-1 expression or activity under physiological and pathological conditions have been extensively studied (Dupont et al., 2009; Jaulmes et al., 2009; Johansson et al., 2000). For example, studies have shown that PAI-1 expression was highly regulated by many factors including cytokines, oxidative stress, and cellular signaling molecules such as mitogen-activated protein kinases (MAPKs) (Dupont et al., 2009; Nerurkar et al., 2007). Our results demonstrated a dose- and time-related increase in the expression of PAI-1 after exposure to non-toxic doses of Nano-CuO. The effects of Nano-CuO on PAI-1 expression effectively altered the balance of the fibrinolytic system that could lead to clot formation. It has been reported that exogenous hydrogen peroxide and endogenous ROS can induce PAI-1 expression in adipocytes, endothelial cells, macrophages, mesangial cells and smooth muscle cells (Jaulmes et al., 2009; Jiang et al., 2003; Chen et al., 2006; Swiatkowska et al., 2002). To investigate the role of ROS in Nano-CuO-induced PAI-1 expression, ROS scavengers or inhibitors such as catalase, DPI, and NAC were used to pre-treat endothelial cells. Our results clearly showed that pre-treatment with catalase, DPI or NAC significantly inhibited Nano-CuO-induced up-regulation of PAI-1 expression.

Under physiological conditions, ROS are produced at low levels that are necessary for maintaining normal cell functions, and the endogenous antioxidant defense systems of the body have the capacity to avert any harmful effects (Donaldson et al., 2003). However, an increase in ROS can itself be considered a type of cellular stress. ROS may also serve as a signal for other kinds of cellular stress (Gough, 2009). ROS mediate activation of MAP kinases in a variety of cells leading to changes in gene expression. In endothelial cells, NF- κ B and AP-1 are two major redox-sensitive transcription factors that are activated through PKC and MAP kinase pathways which respond to oxidative stress (Griendling et al., 2000). Several studies have shown that exposure to various particles, including metal nanoparticles, caused ROS generation leading to the activation of MAP kinases (Mo et al., 2009; Mossman et al., 2006; Wan et al., 2008). Present results have shown that exposure endothelial cells to Nano-CuO caused increased phosphorylation of p38, and the increased phosphorylation of p38 was abolished when cells were pre-treated with ROS inhibitor or scavenger, such as DPI, NAC or catalase. Therefore, ROS generation in endothelial cells with exposure to Nano-CuO is involved in the activation of MAPKs.

Previous studies also showed that activation of p38 MAPK pathway leads to the induction of transcription factors, such as NF- κ B, that is involved in PAI-1 expression (Bonello et al., 2007; Dupont et al., 2009). In this study, we demonstrated that exposure of MPMVEC to Nano-CuO caused up-regulation of PAI-1 in both transcriptional and translational levels. Pre-treatment endothelial cells with ROS inhibitors or scavengers, such as DPI, NAC and Catalase significantly inhibited Nano-CuO-induced up-regulation of PAI-1 expression. Our studies also showed that pre-treatment endothelial cells with the p38 pathway specific inhibitor, SB203580, significantly inhibited PAI-1 up-regulation caused by Nano-CuO. Thus, ROS, generated by exposure endothelial cells to Nano-CuO, resulted in the activation of MAPKs which may further cause impaired endothelial fibrinolysis through increase of PAI-1 expression. Taken together, our findings suggest that Nano-CuO caused ROS generation that mediates the expression of PAI-1 via p38 MAPK pathway.

In conclusion, we have demonstrated that exposure to Nano-CuO caused upregulation of PAI-1 in endothelial cells. The p38 signaling pathway was involved in this process via Nano-CuO-caused oxidative stress. These results provide further understanding of the potential systemic inflammation caused by exposure to metal nanoparticles. The mechanism by which some metal nanoparticles cause increased ROS and subsequent modulation of endothelial fibrinolytic activities remains an important question which warrants further study.

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Abbreviations

ROS	reactive oxygen species
CAT	catalase
NAC	N-acetyl-L(+)-cysteine
DPI	diphenyleneiodonium chloride
DCF	dichlorofluorescein
H ₂ -DCFDA	2', 7'-dichlorodihydrofluorescein diacetate
MAPK	mitogen-activated protein kinase
MPMVEC	mouse pulmonary microvascular endothelial cells
Nano-CuO	nano-size copper (II) oxide
Nano-TiO ₂	nano-size titanium dioxide
PAI-1	plasminogen activator inhibitor-1.

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Fig. 1. Cytotoxicity of Nano-CuO and Nano-TiO₂ on MPMVEC

MPMVEC were treated with different doses of Nano-CuO or Nano-TiO₂ for 24 h, and cytotoxicity was determined by both AlamarBlue™ assay **(A)** and MTS assay **(B)**. MPMVEC without treatment were used as control. Values are mean ± SE of six experiments. * Significant difference from the control group, $p < 0.05$; # Significant difference from the same dose of Nano-TiO₂ group, $p < 0.05$.

Fig. 2. ROS generation in MPMVEC treated with different doses of Nano-CuO (A) and the effects of ROS scavengers or inhibitors on Nano-CuO-induced ROS generation (B) MPMVEC were pre-treated with H₂DCF-DA for 2 h prior to exposure to different doses of Nano-Cu or Nano-TiO₂ for another 12 h (A). For antioxidant experiments, DPI, NAC, or catalase (CAT) was added 2 h prior to adding H₂DCF-DA and then 2.5 µg/ml of Nano-CuO (B). MPMVEC without treatment were used as control. * Significant difference compared with control, $p < 0.01$; # in A, Significant difference from the same dose of Nano-TiO₂-treated group, $p < 0.01$; # in B, Significant difference as compared with Nano-CuO treatment alone, $p < 0.01$.

Fig. 3. Dose-response induction of phosphorylation of p38 in MPMVEC exposed to Nano-CuO MPMVEC were treated with different doses of Nano-CuO or Nano-TiO₂ for 3h. Cytosolic proteins were prepared for Western blot. There is a dose-dependent increase in phosphorylation of p38 (p-p38) when MPMVEC were exposed to Nano-CuO (**A**), but not to Nano-TiO₂ (**B**).

Fig. 4. Time-course of phosphorylation of p38 in MPMVEC exposed to Nano-CuO
 MPMVEC were treated with 2.5 $\mu\text{g/ml}$ of Nano-CuO for 1, 3, 6 and 12h. Cells without treatment were used as control. Cytosolic proteins were subjected to 12% SDS-PAGE. **(A)** shows the result of a single Western blot experiment. **(B)** represents normalized band densitometry readings averaged from 3 independent experiments $\pm$ SE of Western results. The expression level of phospho-p38 (p-p38) was normalized to the expression level of total p38 (p38) in the same sample. 40 μg protein (p-p38 and p38) was loaded in each lane. * Significant difference as compared with the control, $p < 0.05$.

Fig. 5. DPI, NAC or Catalase (CAT) inhibited the increased phosphorylation of p38 in MPMVEC exposed to Nano-CuO

Cells were pretreated with DPI (10 µM), NAC (20 mM) or CAT (1000 U/ml) for 2 h prior to exposure to 2.5 µg/ml of Nano-CuO for another 3 h. Cells without any treatment were used as control. (A) shows the result of a single Western blot experiment. (B) represents normalized band densitometry readings averaged from 3 independent experiments $\pm$ SE of Western-blot results. The expression level of phospho-p38 (p-p38) was normalized to the expression level of total p38 (p38) in the same sample. 40 µg protein was loaded in each lane. * Significant difference as compared with the control, $p < 0.05$; # Significant difference as compared with the Nano-CuO-treated group, $p < 0.05$.

Fig. 6. Dose-response induction of PAI-1 in MPMVEC exposed with Nano-CuO
 Nano-CuO treatment increased PAI-1 mRNA level (3 h) (**A** & **C**) and protein level (6 h) (**B** & **D**) in a dose-dependent manner in MPMVEC. Cells without treatment were used as control. (**A** & **B**) show the result of a single experiment. (**C** & **D**) represent normalized band densitometry readings averaged from 3 independent experiments $\pm$ SE of RT-PCR or Western blot results. * Significant difference as compared with control, $p < 0.05$.

Fig. 7. Time-course induction of PAI-1 mRNA expression in MPMVEC exposed with Nano-CuO MPMVEC were treated with 2.5 µg/ml of Nano-CuO or Nano-TiO₂ for 1, 3, 6 and 12 h. Cells without treatment were used as control. (A) shows the result of a single RT-PCR experiment. (B) represents normalized band densitometry readings averaged from 3 independent experiments ± SE of RT-PCR results. * Significant difference as compared with control, $p < 0.05$; # Significant difference from the same time point of Nano-TiO₂-treated group, $p < 0.05$.

Fig. 8. Effects of ROS inhibitors or scavengers on Nano-CuO-induced expression of PAI-1 in MPMVEC

MPMVEC were pre-treated with catalase (CAT), NAC or DPI for 2 h prior to exposure to 2.5 µg/ml of Nano-CuO for 3 h. Cells without any treatment were used as control. **(A)** shows the result of a single RT-PCR experiment. **(B)** represents normalized band densitometry readings averaged from 3 independent experiments $\pm$ SE of RT-PCR results. * Significant difference as compared with control, $p < 0.05$; # Significant difference as compared with Nano-CuO-treated group, $p < 0.05$.

Fig. 9. The effect of p38 inhibitor on expression of PAI-1 in MPMVEC exposed with Nano-CuO. MPMVEC were pre-treated with the p38 inhibitor, SB203580 (5 or 10 μ M), for 4 h prior to exposure to 2.5 μ g/ml Nano-CuO for another 3 h. Cells without any treatment were used as control. (A) shows the result of a single RT-PCR experiment. (B) represents normalized band densitometry readings averaged from 3 independent experiments $\pm$ SE of RT-PCR results. * Significant difference as compared with control, $p < 0.05$; # Significant difference as compared with Nano-CuO-treated group, $p < 0.05$.

Table 1

Characterization of metal nanoparticles

Metal	Particle size in powder (Diameter) (nm, average)	TEM (nm) Diameter	DLS (nm) Diameter	Specific surface area (m²/g)
Nano-CuO	42	20-40	220	23.0
Nano-TiO ₂	28	10-60	280	45.0