Journal of Toxicology and Environmental Health, Part A, 73:748–756, 2010

ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287391003614042

Taylor & Francis
Taylor & Francis Group

ENDOTOXIN PROMOTES ADVERSE EFFECTS OF AMORPHOUS SILICA NANOPARTICLES ON LUNG EPITHELIAL CELLS IN VITRO

Yongli Shi, Santosh Yadav, Feng Wang, He Wang

Environmental Health Science & Cancer Center, Tulane University, New Orleans, Louisiana, USA

Amorphous silica engineered nanoparticles (ENP) are used for drug delivery and food additive under current regulations. Although the adverse effects of amorphous silica ENP may be negligible, contamination by bacterium products may enhance the toxic potential of these so-called safe products. Lipopolysaccharide (LPS), an endotoxin component generated by gram-negative bacteria, is a potential contaminant of amorphous silica ENP due to its ubiquitous presence in the environment. The combined effects of amorphous silica ENP and LPS are therefore of particular concern. In this study, A549 cells were exposed to amorphous silica ENP in combination with LPS for comparison with the cells treated with ENP. Measurements of MTT assay and lactate dehydrogenase (LDH) activity indicated that the toxicity of amorphous silica ENP was low but co-treatment of the cells with LPS significantly enhanced this toxicity. Decreased cell viability and increased LDH activity release occurred earlier and at lower concentration levels in co-treated cells. Co-treatment of LPS with amorphous silica ENP might also enhance the increase in oxidative stress produced by amorphous silica ENP. However, there were no detectable changes in nitric oxide generation and 8-hydroxy-2-deoxy guanosine formation in the cells treated with either ENP or ENP plus LPS, indicating low effect on oxidative DNA damage. These results showed that LPS may enhance the oxidative stress induced by amorphous silica ENP to initiate cytotoxicity of these engineered nanoparticles.

Nanoparticles (NP) range in size between 1 and 100 nm. Since nanotechnology and related sciences are rapidly developing, exposure to engineered nanoparticles (ENP) is an emerging public health issue. ENP may exhibit size-related properties that differ significantly from those observed in fine or coarse particles (Das et al., 2007; Daniel & Astruc, 2004). The unique physical and chemical properties of ENP attract extensive concern from environmental and occupational health scientists. The diversity of ENP furthers the concern because of unknown health consequences. Importantly, exposure to ENP may occur together with biological hazards such as endotoxin (Vallhov et al., 2006; Ashwood et al., 2007).

Endotoxins are produced by bacteria in the environment or within the human body during infections (Mayeux, 1997). These toxic substances, located in the cell wall of gram-negative bacteria, possess potent biological activities to induce inflammatory reactions. Endotoxins, mainly lipopolysaccharide (LPS), may contaminate ENP (Vallhov et al., 2006). LPS also persists in humans in either clinical or subclinical bacterium infections (Roberts et al., 2008; Kallio et al., 2008; Ara et al., 2009; Hoogerwerf et al., 2009). The presence of LPS in ENP or in human organs poses a medical risk to exposed individuals even if ENP are classified as safe products. The high surface-to-volume ratio of ENP makes the particles reactive, and there is a

Received 23 June 2009; accepted 5 January 2010.
This project is supported by Tulane Cancer Center and Department of Environmental Heath Science, Tulane University.
Address correspondence to He Wang, Environmental Health Science, Tulane University, Tidewater Building, Suite 2129, 1440 Canal Street, New Orleans, LA 70112, USA. E-mail: hwang2@tulane.edu

possibility for LPS to enhance the biological effects of ENP as ENP penetrate through cell membranes, interacting with biological systems, whereas LPS has its receptors on the cell surface to activate biological systems (Mayeux, 1997; Dentener et al., 2000). Combined effects of ENP and endotoxin are likely to occur.

Amorphous silica ENP are widely used industrially. These ENP are also used for drug delivery and food additives. Because of the ubiquitous existence of LPS, combined exposure to amorphous silica ENP and LPS is of particular concern. Generation of oxidative stress due to amorphous silica ENP is well documented (Lin et al., 2006), and it is proposed that oxidative stress is a suitable measure for comparing and discriminating the adverse effects of different ENP (Stone & Donaldson, 2006). Oxidative stress can further lead to inflammatory reactions (Donaldson et al., 2005; BeruBe et al., 2007; Borm et al., 2006). Because LPS is a potent inflammation inducer, the existence of LPS during exposure to amorphous silica ENP may worsen the harmful effects of the particles.

Epithelial cells constitute the first line of physical barriers against invading agents in the lung. Epithelial cells also respond to the invading agents by generating a number of components including reactive oxygen species (ROS) and cytokines. Enhanced oxidative stress in airway epithelial cells was shown in response to exposure to either endotoxin or particles (Becker & Soukup, 1998; Blackford et al., 1997). Previous studies also showed that combination of carbon black (Inoue et al., 2006) or TiO_2 (Inoue et al., 2008) ENP with LPS produced increased adverse effects in lung of experimental animals. Based on these observations, this study was designed to investigate whether LPS might enhance the potential toxicity of amorphous silica ENP in cultured human lung epithelial cells.

MATERIAL AND METHODS

Epithelial Cell Culture

Human type II lung epithelial cell line A549 was purchased from American Type Culture Collection (ATCC) and maintained in Dulbecco's modified Eagle's medium with nutrient mixture (DMEM/F-12) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.2% sodium bicarbonate, 100 U/ml penicillin, and 100 U/ml streptomycin (Sigma Aldrich, St. Louis, MO). Cells were cultured with complete medium at 37°C in 100% humidified atmosphere of 5% CO_2 in air. The cells were free of mycoplasma infection, confirmed by EZ-PCR mycoplasma test kit method (Geneflow, Staffordshire, UK).

Preparation of Amorphous Silica ENP Suspension and LPS Solution

Amorphous silica ENP was purchased from Sigma (Sigma Aldrich, St. Louis, MO). The size is between 10 and 20 nm (measured by transmission electron microscope [TEM] at 200 kV). Other properties were provided by manufacturer (trace metals basis: 99.5%; surface area: BET surface area 140–180 m^2/g). The particles were free of endotoxin, indicated by a high-sensitivity LAL chromogenic endotoxin assay kit (Genscript, Piscataway, NJ) method. The silica stock solution was prepared by dispersing an initial concentration of 1 mg/ml in FBS-free culture medium. In order to prevent agglomeration, the mixture was sonicated for 4 cycles of 20 s on ice and vortexed for 5 min. The stock suspension was kept at 4°C and used within 1 wk for the experiments. Before each treatment, the silica stock suspension was sonicated again as already described, then immediately further diluted to the working solutions with FBS-free culture medium to 5, 50, 100, or 200 μg/ml, respectively.

Lipopolysaccharide (LPS) was purchased from (Sigma Aldrich, St. Louis, MO). The stock solution of LPS was prepared in PBS (pH 7.2) according to the instruction of manufacturer and then further diluted to the working concentration of 100 ng/ml in FBS-free medium for treatment of cultured A549 cells.

Cell Viability Assay

The MTT assay kit (Sigma Aldrich) was used to evaluate the proliferation of A549 cells cultured in media containing different

concentrations of nanoparticles in the absence or presence of LPS (100 ng/ml) according to the manufacturer's instructions. Briefly, A549 cells were seeded into 96-well plates at a cellular density of 5×10^3, 8×10^3, or 1×10^4 cells per well (150 μl/well) to adhere overnight. The wells were washed 3 times with an equal volume of PBS (pH 7.2), and then 150 μl amorphous silica ENP working solution with or without LPS (the final concentration of LPS is 100 ng/ml) was added to each well. The plates were then incubated for 4, 24, or 48 h. At specified time points, 15 μl of 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium (MTT stock solution: 5 mg/ml) was added to each well and incubated for 3 h. After incubation, the medium was removed, and 150 μl MTT solubilization solution was added to the remaining precipitates on the plates in the well. The absorbance of the resulting solution was determined at 550 nm using a microplate reader (BIO-RAD).

Lactate Dehydrogenase Activity Release Assay

Lactate dehydrogenase activity (LDH) release, used as an indicator of cell membrane damage, was measured in the culture medium using an LDH assay kit (Sigma Aldrich, St. Louis, MO) according to the manufacturer's instruction. Total cellular LDH activity was measured in cell lysates obtained by treatment with Triton X-100 solution. Data from control and treated cells were calculated as percent LDH leakage (100 × LDH activity in medium/total LDH activity) and expressed as the mean ± SD, using at least triplicate wells per concentration.

Detection of Intracellular ROS

To measure ROS generation, 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was utilized. Briefly, cells were treated with different concentration of amorphous silica ENP with or without LPS for 24 h. After washing, the cells were incubated with 40 μ*M* DCFH-DA for 45 min in dark. At the end of DCFH-DA incubation, cells were washed with PBS, lysed with 1 *N* NAOH, and aliquots were transferred to a black well plate (BD Falcon). The fluorescent intensity was measured using a multidetection microplate reader (FLUOstar Optima Microplate Reader, BMG LABTECH) with excitation and emission wavelengths of 485 nm and 520 nm, respectively. Three independent experiments were conducted.

GSH Assay

For the GSH assay, a kit method was used (Sigma-Aldrich) according the manufacturer's instructions. Briefly, cells were cultured in media containing different concentrations of amorphous silica ENP with or without LPS at 100 ng/ml for 24 h as described earlier, and the amounts of total glutathione (both oxidized GSSG and reduced GSH, with the GSSG converted to GSH) were measured using a spectrophotometer at 405 nm (MPW-30, series 2600, San Antonio, TX) according to the instruction manual.

Superoxide Dismutase Determination

Superoxide dismutase (SOD) activity of the cultured cells exposed to silica ENP was measured in a classic SOD determination kit (Sigma–Aldrich) according to the manufacturer's instruction.

Nitrate/Nitrite Determination

The levels of nitric oxide in cell culture supernatants were measured by determining the levels of their stable degradation products, nitrate and nitrite, using a nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Nitrate is enzymatically converted into nitrite by the enzyme nitrate reductase and the nitrite was then quantified using Griess reagent. The detection limit of this assay is 2.5 μ*M*.

DNA Oxidative Damage Assay

8-Hydroxy-2-deoxyguanosine (8-OH-dG) serves as an established marker of DNA oxidative damage. In this experiment, levels of 8-OH-dG were measured by an 8-hydroxy-2-deoxy guanosine enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, Ann

Arbor, MI) according to the manufacturer's instructions. Briefly, 50 µl of sample was added to a plate, which was precoated with anti-mouse immunoglobulin (Ig) G and incubated for 18 h at 4ºC. The cells were washed five times to remove all unbound reagents. The color change then was developed with added Ellman's reagent in the dark by using an orbital shaker for 2 h. The absorbance was read at a wavelength of 405 nm. Data were analyzed with Prism software.

Statistical Analysis

All data were expressed as mean ± SD. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA). The analyses were combined with Tukey's test to compare all pairs of columns for discovering where the significant differences occurred. In all the tests, statistical significance was set at a p value less than .05.

RESULTS

Cell Viability Test by MTT Assay

A549 cells were treated with various concentrations of silica ENP suspensions for different periods. Cell viability decreased as concentration increased. At a concentration of 200 µg/ml, cells treated for 4 h showed a significant decrease in cell viability in comparison with vehicle control group (Figure 1). At 48 h, high-concentration silica ENP suspension (100 µg/ml and 200 µg/ml) produced significantly decreased cell viability relative to control. As shown in Figure 1, decreased cell viability occurred in concentration- and time-dependent manners after treatment with amorphous silica ENP.

As shown in Figure 2, when the preceding test was repeated with addition of LPS at a concentration of 100 µg/ml, significantly decreased cell viability appeared at all particle concentrations and time points except low-concentration silica, 5 µg/ml, at 24 h treatment time duration. LPS accelerated and enhanced the appearance of cytotoxicity of silica ENP, indicating a combined effect on the cell viability.

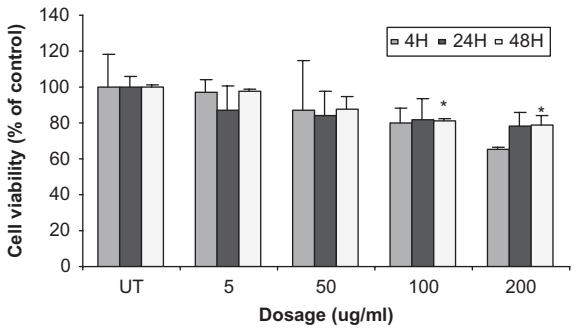

FIGURE 1. Cell viability in A549 cells treated with increasing concentrations of amorphous silica engineered nanoparticles (expressed as percent of control) at 4, 24, or 48 h of incubation as determined by MTT assay. UT means treated with vehicle only. Asterisk indicates significant at $p < .05$ versus control.

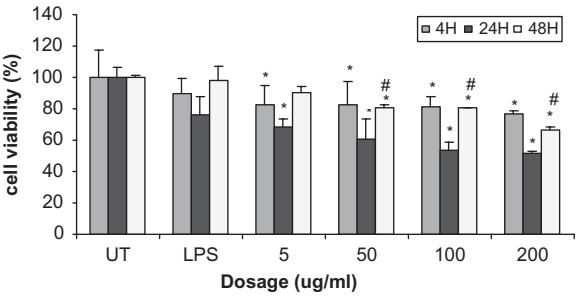

FIGURE 2. Cell viability in A549 cells treated with increasing concentrations of amorphous silica engineered nanoparticles plus 100 ng/ml LPS in each group at 4, 24, or 48 h of incubation as measured by MTT assay. Data expressed as percent of control. UT means treated with vehicle only; LPS means treated with LPS only at 100 ng/ml concentration; 5, 50, 100, or 200 µg/ml in this figure means LPS (100 ng/ml) plus the corresponding level of amorphous silica ENP. Asterisk indicates significant at $p < .05$ versus control; #, significant at $p < .05$ versus LPS group.

LDH Activity Release

LDH activity levels increased in the groups treated with rising concentrations of amorphous silica ENP. The rise occurred in a concentration- and time-dependent manner as shown in Figure 3(A), although statistical significance was not reached. In the groups co-treated with both LPS (100 ng/ml) and amorphous silica ENP, however, the elevated release of LDH, as shown in Figure 3(B), occurred earlier and the increases reached significance at 24 h.

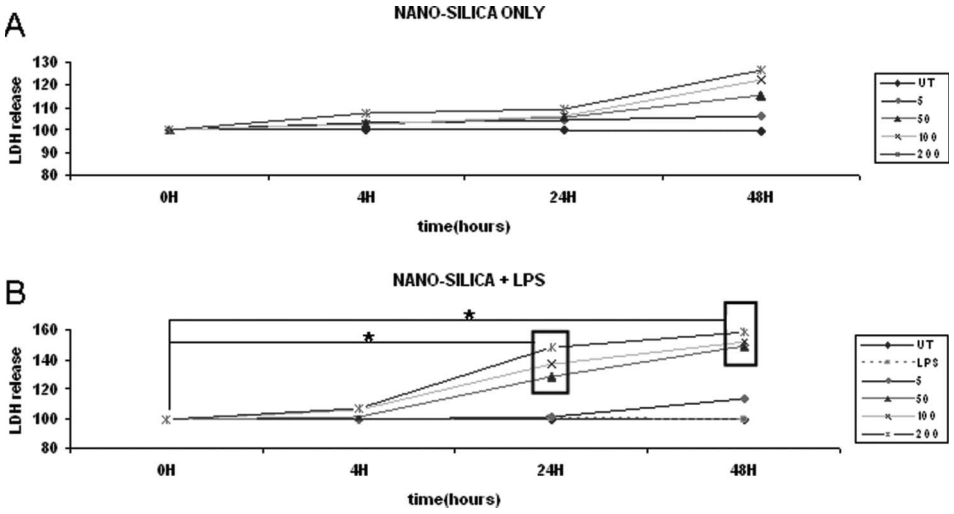

FIGURE 3. LDH release from A549 cells treated with different concentrations of amorphous silica ENP without (A) or with 100 ng/ml LPS (B). Data are expressed as percent of extracellular LDH. Asterisk indicates significant at $p < .05$ versus control. UT means treated with vehicle only; LPS means treated with 100 ng/ml LPS; the concentrations of amorphous silica ENP in (B) mean 100 ng/ml plus the corresponding level of particle concentration.

ROS Levels

Cells were treated with amorphous silica ENP at concentrations of 0, 5, 50, 100, or 200 μg/ml with (S + LPS) or without (S) LPS (100 ng/ml) for 24 h. This time duration was selected because of the significant decrease in cell viability and increase in LDH activity levels at this time point. After 24 h of incubation, ROS levels were determined. As shown in Figure 4, treatment with amorphous silica ENP alone stimulated the generation of intracellular ROS in a concentration-dependent manner. Significant increase appeared at 100 μg/ml. When co-treated with LPS, ROS rise occurred at 50 μg/ml ENP.

GSH Levels

As ROS levels increased, the effect of LPS and amorphous silica ENP on antioxidants was also tested by measuring GSH and SOD. The levels of GSH in the amorphous silica ENP group decreased in a concentration-dependent manner after treatment with increasing concentrations of amorphous silica EN. Significant decrease appeared at 50 μg/ml ENP. LPS (100 ng/ml) alone also decreased GSH significantly and fall occurred in a concentration-dependent manner after addition of increasing ENP amounts, as shown in Figure 5.

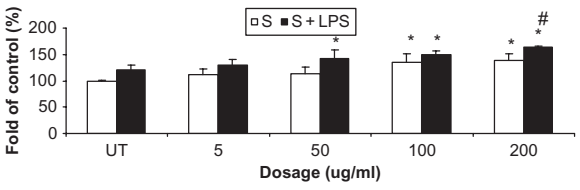

FIGURE 4. Effects of amorphous silica ENP without or with LPS on the generation of ROS from A549 cells at different concentrations. Data are expressed as percent ROS level of control. Asterisk indicates significant at $p < .05$ versus control; #, significant at $p < .05$ versus LPS. S means silica treatment groups; S + LPS means silica plus LPS 100 ng/ml groups.

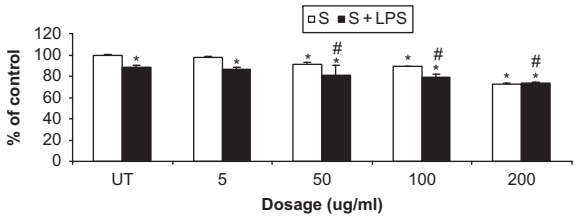

FIGURE 5. Effects of amorphous silica ENP at different concentrations without or with LPS on levels of GSH in A549 cells. Data are expressed as percent of the GSH level in control group. Asterisk indicates significant at $p < .05$ versus control; #, significant at $p < .05$ versus LPS group. S means silica treatment groups; S + LPS means silica plus LPS 100 ng/ml groups.

SOD Activity Levels

As shown in Figure 6, SOD activity levels were also lowered by treatment with increasing concentrations of amorphous silica ENP without LPS. A significant decrease appeared at 200 μg/ml ENP. Treatment of LPS only at the level of 100 ng/ml significantly lowered the SOD levels in A549 cells. Addition of amorphous silica ENP did not further affect SOD, indicating a threshold for SOD activity decrease by LPS.

Nitric Oxide Production

Nitric oxide (NO) production represented by nitrate/nitrite level in cell culture supernatant was determined by ELISA. As shown in Figure 7, all the OD values of different treatment groups were lower than for the concentration of 5 μ*M* from the standard curve.

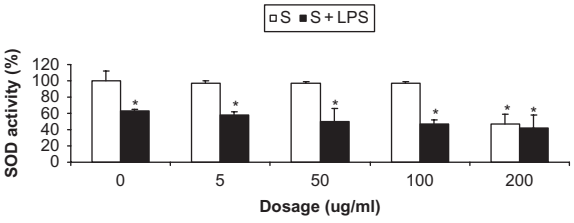

FIGURE 6. Effects of amorphous silica ENP at different concentrations without or with LPS on the activity of SOD from A549 cells. Data are expressed as percent of SOD activity in control group. Asterisk indicates significant at $p < .05$ versus control (treated with vehicle only). S means silica treatment groups; S + LPS means silica plus LPS 100 ng/ml groups.

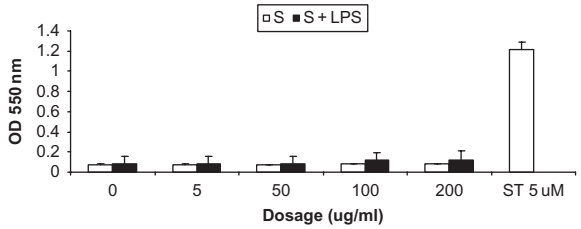

FIGURE 7. Effect of amorphous silica ENP on the production of nitric oxide in A549 cells treated with increasing concentrations of the particles with or without LPS. ST 5 μ*M* means the OD standard 5 μ*M* nitrate/nitric. S means amorphous silica treatment groups; S + LPS means LPS (100 ng/ml) plus silica treatment groups.

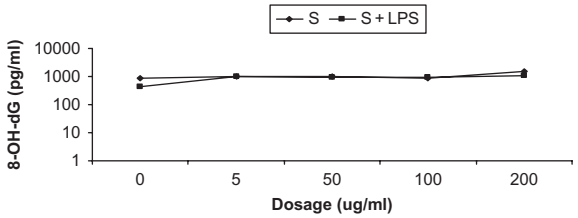

FIGURE 8. Effect of amorphous silica ENP on the level of 8-OH-dG supernatant of cultured A549 cells treated with increasing concentrations of the particles with or without LPS. S means amorphous silica treatment groups; S + LPS means LPS (100 ng/ml) plus silica treatment groups.

DNA Oxidative Damage/8-OH-dG Level in Cell Culture Supernatant

The supernatant of cell culture after treating with amorphous silica ENP with or without LPS (100 ng/ml) was used for measurement of 8-OHdG, an oxidative DNA damage marker. As shown in Figure 8, there was no significant change in levels of 8-OH-dG in cell culture supernatant in different treatment groups.

DISCUSSION

It is well known that LPS is an endotoxin that induces a potent response in the normal animal immune system (Mayeux, 1997). LPS binds the CD14/TLR4/MD2 receptor complex in macrophages to induce secretion of inflammatory cytokines. Interestingly, cultured type II epithelial cells, A549 cell line, possibly have CD14 on cell surface (Cheon et al., 2008). Zhou et al. (2008) demonstrated that A549 cells contain TLR4 receptors and LPS binding protein (Dentener et al., 2000). Lung epithelial cells are in the front line against invading agents; thus, the interaction between LPS and type II epithelial cells is likely to occur via this pathway. Amorphous silica ENP was reported to produce increased oxidative stress in A549 cells (Lin et al., 2006). It was postulated that that LPS, a potential contaminant of ENP, enhances airway epithelial cell damage in combination with amorphous silica ENP by furthering ENP-induced oxidative stress.

In this study, an LPS concentration of 100 ng/ml was selected that did not induce significant

cell death and cell membrane damage for co-treatment with amorphous silica ENP. This is because there is no available data regarding the amount of LPS in ENP and LPS levels in humans. A549 cells were selected because these cells have the properties of type II alveolar epithelial cells and are widely used. In this study, A549 cells were exposed to both LPS and amorphous silica ENP for comparison with toxicity produced by exposure to amorphous silica ENP alone. Although the amorphous silica decreased the cell viability of A549 cells, the combined exposure of low-level LPS and amorphous silica ENP modified cell reactions with regard to both timing and concentration. Significant decrease in cell viability occurred earlier in groups with combined exposure compared to ENP alone. The significant decrease in cell viability also occurred at a much lower concentration in the groups with combined exposure. LDH activities, representing damage to cell membrane, also occurred in a similar pattern with respect to cell viability.

A549 cells express TLR4 and directly respond to LPS (MacRedmond et al., 2005; Wen et al., 2009; Zhou et al., 2008). A549 cells may use soluble CD14 as TLR co-receptor (Regueiro et al., 2006). 52K 10 is a gram-negative bacterium and LPS can therefore be generated by it. Infection of A549 cells in vitro by this bacterium may trigger inflammatory reactions, possibly via the CD14-TLR pathway (Regueiro et al., 2006). In our experiment, direct exposure of A549 cells to LPS at the selected concentration (100 ng/ml) actually quantitatively increased intracellular ROS. LPS treatment of A549 cells also reduced the levels of antioxidants SOD and glutathione significantly in these cells. The decreases in antioxidants and increases in ROS may at least partially contribute to the earlier and greater cell damage produced by coexposure to LPS and amorphous silica ENP.

SOD protects cells from oxidative stress (Vincent et al., 2009). It was noted that LPS at 100 ng/ml lowered SOD significantly and exposure to ENP did not further decrease SOD levels. Amorphous silica ENP reduced the levels of GSH (Lin et al., 2006). In this experiment, GSH levels in LPS exposed cells were reduced and further lowered by ENP addition. ROS generation occurred in a similar pattern to GSH. At the selected LPS concentration, it might have reached a threshold for SOD decrease, but not for the increase of ROS production and glutathione consumption. Further generation of ROS and decrease in GSH levels may actually have produced damage to A549 cells with combined exposure. In addition, no changes in NO generation were detected in A549 cells exposed to either LPS or LPS plus ENP. Similarly, significant oxidative DNA damage was not seen in these cells.

Recently, Park and Park (2009) demonstrated that amorphous silica nanoparticles induced pro-inflammatory responses in vivo/in vitro, and the pro-inflammatory responses may be triggered by ROS generation. The exposure to high concentrations of amorphous silica nanoparticles might be toxic to A549 cells (Chang et al., 2007; Lin et al., 2006), and nanosized amorphous silica induced ROS in A549cells (Lin et al., 2006). However, the dye-doped luminescent silica nanomaterials exert low genotoxicity and cytotoxicity in A549 cells (Jin et al., 2007). In this study, LPS with amorphous silica ENP produced not only increased ROS generation but also decreased levels of antioxidants. It is possible that the increased ROS and decreased antioxidants trigger inflammatory responses in human alveolar epithelial cells in vivo. Further studies are needed to confirm this speculation, although ENP such as carbon black (Inoue et al., 2006) and TiO_2 (Inoue et al., 2008) as well as diesel exhaust nanoparticles (Inoue et al., 2007) produced enhanced lung inflammation by LPS in animal models.

In conclusion, this study demonstrated that cytotoxicity of amorphous silica ENP is enhanced significantly by coexposure with LPS in type II alveolar epithelial cells. Because epithelial cells constitute the first line of defense against airborne hazards, contamination of amorphous silica ENP by LPS or infection of gram-negative bacteria may result in serious consequences to exposed subjects. The ubiquitous presence of LPS and subclinical bacterium infections increase the likelihood of

coexposure. In production of amorphous ENP and utilization of these particles, especially in drug and food industries as well as in clinical settings, strict control and testing for LPS need to be adopted. The underlying mechanism for LPS to enhance the cytotoxicity of amorphous silica ENP may be attributed to its induction of oxidative stress in alveolar type II epithelial cells. However, further studies are needed for direct evidence of this mechanism.

REFERENCES

Ara, T., Kurata, K., Hirai, K., Uchihashi, T., Uematsu, T., Imamura, Y., Furusawa, K., Kurihara, S., and Wang, P. L. 2009. Human gingival fibroblasts are critical in sustaining inflammation in periodontal disease. *J. Periodontal Res*. 44:21–27.

Ashwood, P., Thompson, R. P., and Powell, J. J. 2007. Fine particles that adsorb lipopolysaccharide via bridging calcium cations may mimic bacterial pathogenicity towards cells. *Exp. Biol. Med. (Maywood)* 232:107–117.

Becker, S., and J. M. Soukup. 1998. Decreased CD11b expression, phagocytosis, and oxidative burst in urban particulate pollution-exposed human monocytes and alveolar macrophages. *J. Toxicol Environ Health A* 55:455–477.

BeruBe, K., Balharry, D., Sexton, K., Koshy, L., and Jones, T. 2007. Combustion-derived nanoparticles: mechanisms of pulmonary toxicity. *Clin. Exp. Pharmacol. Physiol.* 34:1044–1050.

Blackford, J. A., Jr., Jones, W., Dey, R. D., and Castranova, V. 1997. Comparison of inducible nitric oxide synthase gene expression and lung inflammation following intratracheal instillation of silica, coal, carbonyl iron, or titanium dioxide in rats. *J. Toxicol. Environ. Health* 51:203–218.

Borm, P. J., Robbins, D., Haubold, S., Kuhlbusch, T., Fissan, H., Donaldson, K., Schins, R., Stone, V., Kreyling, W., Lademann, J., Krutmann, J., Warheit, D., and Oberdorster, E. 2006. The potential risks of nanomaterials: a review carried out for ECETOC. *Part. Fibre Toxicol.* 3:11.

Chang, J. S., Chang, K. L., Hwang, D. F., and Kong, Z. L. 2007. In vitro cytotoxicitiy of silica nanoparticles at high concentrations strongly depends on the metabolic activity type of the cell line. *Environ. Sci. Technol.* 41:2064–2068.

Cheon, I. S., Woo, S. S., Kang, S. S., Im, J., Yun, C. H., Chung, D. K., Park, D. K., and Han, S. H. 2008. Peptidoglycan-mediated IL-8 expression in human alveolar type II epithelial cells requires lipid raft formation and MAPK activation. *Mol. Immunol.* 45:1665–1673.

Daniel, M. C., and Astruc, D. 2004. Gold nanoparticles: Assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology. *Chem. Rev.* 104:293–346.

Das, S., Basu, S., Majumdar, G., Chakravorty, D., and Chaudhuri, S. 2007. Sol-gel synthesized SnO_2 nanoparticles and their properties. *J. Nanosci. Nanotechnol.* 7:4402–4411.

Dentener, M. A., Vreugdenhil, A. C., Hoet, P. H., Vernooy, J. H., Nieman, F. H., Heumann, D., Janssen, Y. M., Buurman, W. A., and Wouters, E. F. 2000. Production of the acute-phase protein lipopolysaccharide-binding protein by respiratory type II epithelial cells: Implications for local defense to bacterial endotoxins. *Am. J. Respir. Cell Mol. Biol.* 23:146–153.

Donaldson, K., Tran, L., Jimenez, L. A., Duffin, R., Newby, D. E., Mills, N., MacNee, W., and Stone, V. 2005. Combustion-derived nanoparticles: A review of their toxicology following inhalation exposure. *Part. Fibre Toxicol.* 2:10.

Hoogerwerf, J. J., de Vos, A. F., Levi, M., Bresser, P., van der Zee, J. S., Draing, C., von Aulock, S., and van der Poll, T. 2009. Activation of coagulation and inhibition of fibrinolysis in the human lung on bronchial instillation of lipoteichoic acid and lipopolysaccharide. *Crit. Care Med.* 37:619–625.

Inoue, K., Takano, H., Ohnuki, M., Yanagisawa, R., Sakurai, M., Shimada, A., Mizushima, K., and Yoshikawa, T. 2008. Size effects of nanomaterials on lung inflammation and coagulatory disturbance. *Int. J. Immunopathol. Pharmacol.* 21:197–206.

Inoue, K., Takano, H., Yanagisawa, R., Hirano, S., Kobayashi, T., Fujitani, Y., Shimada, A., and Yoshikawa, T. 2007. Effects of inhaled nanoparticles on acute lung injury induced by lipopolysaccharide in mice. *Toxicology* 238:99–110.

Inoue, K., Takano, H., Yanagisawa, R., Hirano, S., Sakurai, M., Shimada, A., and Yoshikawa, T. 2006. Effects of airway exposure to nanoparticles on lung inflammation induced by bacterial endotoxin in mice. *Environ. Health Perspect.* 114:1325–1330.

Jin, Y., Kannan, S., Wu, M., and Zhao, J. X. 2007. Toxicity of luminescent silica nanoparticles to living cells. *Chem. Res. Toxicol.* 20:1126–1133.

Kallio, K. A., Buhlin, K., Jauhiainen, M., Keva, R., Tuomainen, A. M., Klinge, B., Gustafsson, A., and Pussinen, P. J. 2008. Lipopolysaccharide associates with pro-atherogenic lipoproteins in periodontitis patients. *Innate Immun.* 14:247–253.

Lin, W., Huang, Y. W., Zhou, X. D., and Ma, Y. 2006. In vitro toxicity of silica nanoparticles in human lung cancer cells. *Toxicol. Appl. Pharmacol.* 217:252–259.

MacRedmond, R., Greene, C., Taggart, C. C., McElvaney, N., and O'Neill, S. 2005. Respirator epithelial cells require Toll-like receptor 4 for induction of human beta-defensin 2 by lipopolysaccharide. *Respir. Res.* 6:116.

Mayeux, P. R. 1997. Pathobiology of lipopolysaccharide. *J. Toxicol. Environ. Health* 51:415–435.

Park, E. J., and Park, K. 2009. Oxidative stress and pro-inflammatory responses induced by silica nanoparticles in vivo and in vitro. *Toxicol. Lett.* 184:18–25.

Regueiro, V., Campos, M. A., Pons, J., Alberti, S., and Bengoechea, J. A. 2006. The uptake of a *Klebsiella pneumoniae* capsule polysaccharide mutant triggers an inflammatory response by human airway epithelial cells. *Microbiology* 152:555–566.

Roberts, H. C., Moseley, R., Sloan, A. J., Youde, S. J., and Waddington, R. J. 2008. Lipopolysaccharide alters decorin and biglycan synthesis in rat alveolar bone osteoblasts: Consequences for bone repair during periodontal disease. *Eur. J. Oral Sci.* 116: 207–216.

Stone, V., and Donaldson, K. 2006. Nanotoxicology: Signs of stress. *Nat Nanotechnol* 1:23–24.

Vallhov, H., Qin, J., Johansson, S. M., Ahlborg, N., Muhammed, M. A., Scheynius, A., and Gabrielsson, S. 2006. The importance of an endotoxin-free environment during the production of nanoparticles used in medical applications. *Nano Lett.* 6:1682–1686.

Vincent, A., Babu, S., Heckert, E., Dowding, J., Hirst, S. M., Inerbaev, T. M., Self, W. T., Reilly, C. M., Masunov, A. E., Rahman, T. S., and Seal, S. 2009. Protonated nanoparticle surface governing ligand tethering and cellular targeting. *ACS Nano 3*:1203–1211.

Wen, X., Chen, X., and Zhou, X. 2009. [Rebamipide inhibited expression of TLR4 and TNF alpha release in pulmonary epithelial cell line A549 induced by lipopolysaccharide.]. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 34:457–160.

Zhou, M., Wan, H. Y., Huang, S. G., Li, B., and Li, M. 2008. [Expression of toll-like receptor 4 in human alveolar epithelial cells and its role in cellular inflammation]. *Zhonghua Yi Xue Za Zhi* 88:2112–2116.