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Inhalation Exposure to Nickel Hydroxide Nanoparticles Induces Systemic Acute Phase Response in Mice

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It has been proposed that acute phase response can be a mechanism by which inhaled particles exert adverse effects on the cardiovascular system. Although some of the human acute phase proteins have been widely studied as biomarkers of systemic inflammation or cardiovascular diseases, there are only a few studies that investigated the role of serum amyloid P (SAP), a major acute phase protein in mice. In this study, we investigated the changes in SAP, following inhalation exposure to nickel hydroxide nanoparticles (nano-NH). We conducted 1) acute (4 h) exposure to nano-NH at 100, 500, and 1000 $\mu g/m^3$ and 2) sub-acute (4h/d for 3d) exposure at 1000 $\mu g/m^3$, then measured serum SAP protein levels along with hepatic *Sap* mRNA levels. The results show that inhaled nano-NH can induce systemic acute phase response indicated by increased serum SAP levels and hepatic *Sap* mRNA levels. To the best of our knowledge, this is the first study showing induction of SAP in response to repeated particle exposure, and the results suggest that SAP can be used as a biomarker for systemic inflammation induced by inhaled particles.

***Key words*:** Nickel hydroxide nanoparticles, Acute phase response, Serum amyloid P component, Inhalation, Systemic inflammation

INTRODUCTION

Epidemiological studies have suggested an association between respiratory exposure to ambient particles and increased risk of cardiovascular diseases (Pope *et al.*, 2004). Although the underlying mechanisms of this association are yet to be determined, it has been suggested that particle exposure may cause cardiovascular effects through particle-mediated pulmonary inflammation that further leads to systemic inflammation (Saber *et al*., 2009).

One of the mechanisms proposed to play a role in particle-induced systemic inflammation is acute phase response (Donaldson *et al*., 2001). Acute phase response is a non-specific innate immune response to various stimuli including stress, injury, infection and inflammation, and the proteins whose plasma levels are increased during the acute phase response are called acute phase proteins (Cray *et al*., 2009). Acute phase proteins have been well recognized for their applications in the diagnosis and prognosis of cardiovascular diseases (Cray *et al.*, 2009), and especially C-reactive protein (CRP), the most well-known acute phase protein, has been widely studied as a potential biomarker for cardiovascular diseases in human (de Ferranti and Rifai, 2007). For this reason, various studies have investigated the role of CRP in particle-mediated systemic inflammation, and found that exposure to ambient particles increase blood C-reactive protein (CRP) levels in humans (Delfino *et al.*, 2008; Ohlson *et al.*, 2010; Pope *et al.*, 2004; Ruckerl *et al.*, 2006) and rats (Lei *et al.*, 2005; Niwa *et al.*, 2008).

Despite of the high level of interest in CRP, there are only a few studies that have investigated serum amyloid P (SAP), the mouse equivalent of CRP, as a marker of systemic inflammation. Therefore, in this study, we investigated whether SAP could be induced in mice following inhalation exposure to particles. As a test material, we used nickel hydroxide nanoparticles (nano-NH), a manufactured nanomaterial of growing interest in power/energy industry (Rocha *et al.*, 2009).

First, we exposed mice to nano-NH at three different con-

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Abbreviations: ApoE: apoprotein E, CRP: C-reactive protein, FA: Filtered Air, LDL: Low density lipoprotein, nano-NH: nickel hydroxide nanoparticles, OSHA: Occupational Safety and Health Administration, PEL: Permissible Exposure Limit, SAP: Serum Amyloid P component

centrations (100, 500, 1000 μg/m^3) for 4 h and measured serum SAP protein levels. We also determined hepatic *Sap* mRNA levels, because acute phase proteins are mainly produced by the liver and regulated at the transcriptional level (Ferri *et al.*, 2007). In addition, we conducted a 3-day (3d) exposure to 1000 μg/m^3 of nano-NH and measured the same endpoints. The results show that exposure to nano-NH can induce acute phase response in mice, as reflected in increased hepatic *Sap* mRNA levels as well as elevated serum SAP levels.

MATERIALS AND METHODS

Animals. Three-month-old male C57BL/6 mice were obtained from Taconic Farms (Germantown, NY) and housed in our "Association for Assessment and Accreditation of Laboratory Animal Care"-accredited animal facility. Animals were provided with standard rodent chow (Harlan Teklad diet, Indianapolis, IN) and water *ad libitum*, and had at least a 2-week acclimation period before being used for experiments. All procedures involving animals were conducted in compliance with guidelines for ethical animal research and approved by the New York University School of Medicine Animal Care and Use Committee.

Exposure and sacrifice protocol. Animals (n = 5/group) were exposed to nickel hydroxide nanoparticles (nano-NH) via a whole body inhalation system, and details of particle generation and the exposure system using this method were described in a previous publication (Gillespie *et al.*, 2010). The nominal exposure concentrations were set at 100, 500, and 1000 μg/m^3 for 1 day (1d) study (single exposure for 4 h), and 3d exposure experiment was conducted at 1000 μg/m^3 for 4 h/d for three consecutive days. Control animals were placed in an identical exposure chamber but received only filtered compressed air (FA). Mice were sacrificed by an overdose of sodium pentobarbital (150~200 mg/kg) via intraperitoneal injection, at 24 h after the exposure (the last exposure in the case of 3d study). A 24 h time-point was chosen because the change in plasma concentration of major acute phase proteins peak around 24 h after stimuli (Gabay and Kushner, 1999) and rapidly decline due to their short half-life (Cray *et al.*, 2009).

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Transcriptional changes of *Sap* gene were evaluated using 10~25 mg of the liver tissue (median lobe). The tissue was harvested at sacrifice and stored in RNALater (ambion, Austin, TX) at -20°C until further processing. RT-PCR was conducted following the procedure described in our previous publication (Gillespie *et al.*, 2010) and relative expression levels of *Sap* gene were normalized to the housekeeping gene, hypoxanthine phosphoribosyltransferase (*Hprt-1*) and reported as relative fold changes over control group.

Serum SAP measurement. Whole blood was collected at sacrifice by heart puncture and, after 20 min incubation at room temperature for clotting, serum was separated by centrifugation at 2000 rpm for 10 min. Serum samples were stored at -80°C and analyzed for SAP using a commercial Enzyme Linked ImmunoSorbent Assay (ELISA) kit (Kamiya biomedical company, Seattle, WA).

Nickel content analyses. To determine whether changes in hepatic *Sap* mRNA are results of direct toxicity from translocated nano-NH, Ni contents in the liver were analyzed. The liver was removed from each animal, weighed and wet-ashed in Teflon beakers using optima grade nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) (both from Fisher Scientific, Pittsburgh, PA). The nickel contents in the samples were measured using graphite furnace-atomic absorption spectroscopy (GF-AAS, GF95, Thermo Scientific, Waltham, MA).

Statistical analyses. All data are expressed as mean ± standard error of the mean (SEM), unless otherwise specified. One-way analysis of variance (ANOVA) accompanied by post-hoc tests or Student's t-test was used to compare differences among test groups, and a p-value less than 0.05 was considered significant. All statistical analyses were performed using Graphpad Prism software (Version 5, Graphpad Software Inc, San Diego, CA).

RESULTS

Nano-NH exposure. Nano-NH were mostly agglomerates with the count median diameter of approximately 40 nm and the diameter of primary particle was found to be 5 nm. Detailed particle characteristics including Transmission Electron Microscope (TEM) images and particle number concentrations were described in our previous study (Gillespie *et al.*, 2010). For 1-day experiment, the nominal exposure concentrations were set at 100, 500, and 1000 μg/

Table 1. Exposure concentrations of nano-NH used in this study

	Nominal total mass conc. (μg/m^3)	Actual total mass conc. (μg/m^3)	Nickel mass conc. (μg/m^3)[a]	Exposure time
NH-100	100	103.2	65.4	4h
NH-500	500	565.0	358.2	4h
NH-1000	1000	1204.0	763.3	4h
3d NH-1000	1000	1062.5 ± 65.8[b]	673.6 ± 41.7	4h x 3d

Conc., concentration.
[a]Nickel mass conc. was calculated based on the molecular weight ratio of Ni to $Ni(OH)_2$.
[b]Values are the average ± standard deviation of the concentrations measured on each day during the 3d experiment.

m^3 of nano-NH (as indicated NH-100, NH-500, and NH-1000, respectively), and the actual concentration for each group was shown in Table 1. Nickel mass concentration was calculated based on the molecular weight ratio of Ni to $Ni(OH)_2$ (59/93 = 63.4%). The average actual mass concentration for 3d exposure at 1000 μg/m^3 of nano-NH was 1062.5 ± 65.8 μg/m^3. It is worth noting that these exposure concentrations are below the current Occupational Safety and Health Administration (OSHA)'s Permissible Exposure Limit (PEL) for nickel hydroxide, 1 mg Ni/m^3 (TOXNET Toxicology Data Network, 2003).

1-day exposure. Fig. 1A shows that serum SAP levels increased significantly after inhalation exposure to 1000 μg/m^3 of nano-NH, the highest concentration tested in this study. Although slight differences were found in NH-100 and NH-500 groups compared with the control group, they were not statistically significant. Consistently, *Sap* mRNA amount in the liver were significantly higher only in the NH-1000 group (2.9 fold; Fig. 2B). These results indicate that acute exposure to inhaled nano-NH at levels below the current OSHA PEL can induce acute phase response in mice.

3-day exposure. To determine the effects of sub-acute exposure to nano-NH on acute phase response, mice were exposed to 1000 μg/m^3 of nano-NH for 4 h/d for three consecutive days (3d NH-1000). As shown in Fig. 2A, serum SAP levels of the exposed mice increased even further to 34.6 μg/m*l*. The hepatic *Sap* mRNA level was still significantly higher compared with the control group, although it was lower than that of 1d NH-1000 group (1.5 fold vs 2.9 fold) (Fig. 2B).

Nickel contents in the liver. To determine whether hepatic mRNA changes are induced by direct toxicity by translocated or metabolized Ni in the liver, we measured Ni contents in the tissue. For 1d study, there was no difference in Ni contents in the liver, although dose-dependent lung burden was reported in our previous study (Gillespie *et al.*,

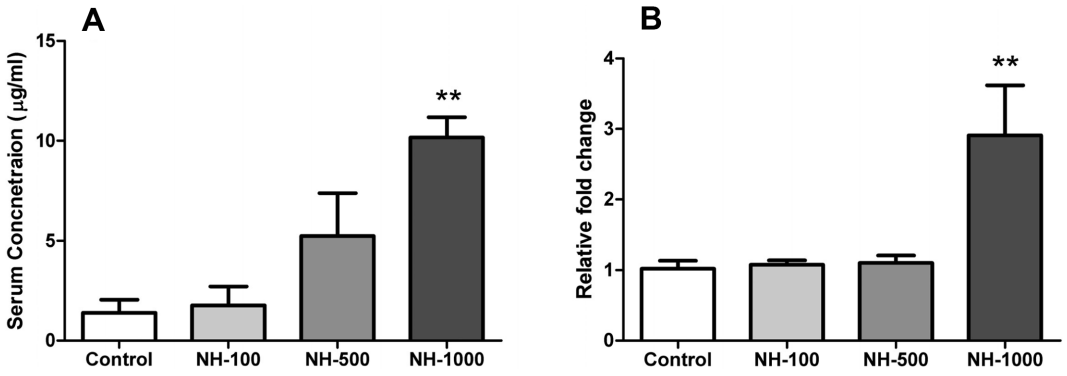

Fig. 1. A) Serum SAP concentrations and B) Relative hepatic *Sap* mRNA levels; in mice exposed to FA or nano-NH at 100, 500, 1000 μg/m^3 for 4 h. Values are mean ± SEM. *p < 0.05 and **p < 0.01 compared with control.

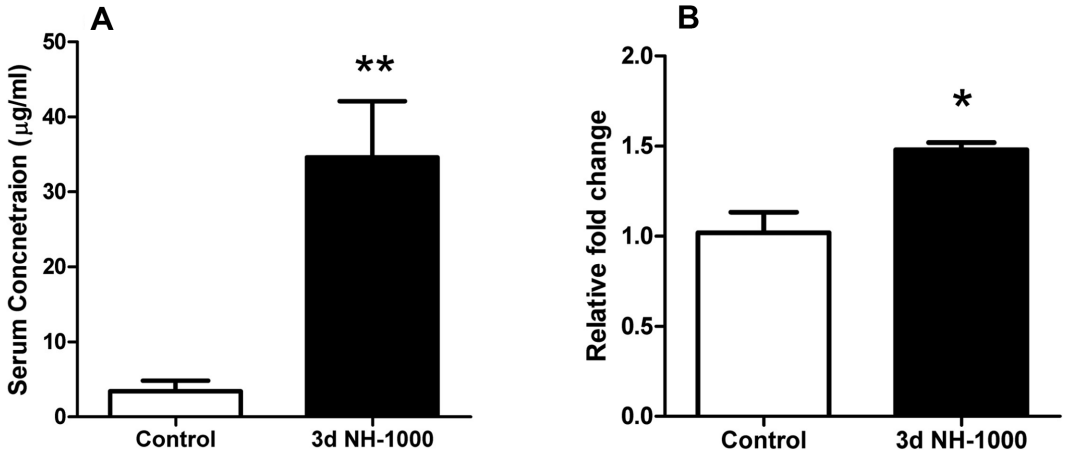

Fig. 2. A) Serum SAP concentrations and B) Relative hepatic *Sap* mRNA levels; in mice at 24 h after the last exposure to FA or nano-NH at 1000 μg/m^3 for 4 h/d for 3d. Values are mean ± SEM. *p < 0.05 and **p < 0.01 compared with control.

2010). Consistently, 3d exposure did not result in significant difference in liver Ni contents, either. These data indicate that the observed transcriptional changes in the liver are the results of systemic inflammation originated from the lung.

DISCUSSION

In the last decade, various studies have reported that pulmonary exposure to particles can induce acute phase response in humans and rats (Delfino *et al.*, 2008; Ohlson *et al.*, 2010; Pope *et al.*, 2004; Ruckerl *et al.*, 2006; Lei *et al.*, 2005; Niwa *et al.*, 2008). Moreover, it has been suggested that this particle-induced acute phase response may further affect the cardiovascular system (Donaldson *et al.*, 2005). However, studies on mouse acute phase response following particle exposure are relatively rare, and there are only a few studies investigated SAP, a major acute phase protein in mouse, as a potential marker for systemic inflammation or cardiovascular health. Our previous study has shown that long-term exposure to inhaled nano-NH can induce systemic inflammation and accelerated atherosclerosis in a susceptible mouse model (Kang *et al.*, 2011). Therefore, in this study, we investigated whether acute inhalation exposure to nano-NH can induce SAP in wild-type mice.

The results clearly show that acute exposure to inhaled nano-NH can induce acute phase response in mice, marked by significant increase in both serum protein concentration and hepatic mRNA level of SAP. The data from Ni content analyses suggest that these changes are not due to the direct toxicity of Ni accumulated in the liver. As reported in our previous study, NH-1000 exposure induced significant pulmonary inflammation in the same mice (Gillespie *et al.*, 2010). Thus, it is more likely that the aforementioned changes in SAP are indication of systemic inflammation that stemmed from pulmonary inflammation following nano-NH exposure.

Most acute phase proteins including SAP are produced mainly by hepatocytes following various stimuli including infection and inflammation (Cray *et al.*, 2009), and their production is regulated primarily at the transcriptional level (Saber *et al.*, 2009). However, most studies investigating association between particle exposure and induction of acute phase response are based on acute phase protein levels in the blood (Saber *et al.*, 2009). For this reason, in addition to serum SAP concentrations, we measured changes in the hepatic *Sap* mRNA levels to examine whether they could be used as a sensitive marker of acute phase response.

To the best of our knowledge, this is the first study showing SAP induction by repeated particle exposures at both hepatic mRNA level and blood concentration level. Saber *et al.*, measured these endpoints in response to diesel exhaust particles or carbon black, but found no significant change despite pulmonary inflammation (Saber *et al.*, 2009). In our previous study, we showed that hepatic *Sap* mRNA levels increased significantly after exposure to low level of nano-NH (~125 μg/m^3) for 1w (3.5-fold) and 5 m (3.6-fold) in a hyperlipidemic apoprotein E-deficient (ApoE$^{-/-}$) mouse model (Kang *et al.*, 2011). However, the protein levels of SAP in the serum were not significantly different between control and nano-NH-exposed mice.

It was especially interesting for us to find changes in both hepatic mRNA levels and serum protein concentrations in this study, because, as indicated above, there was discrepancy between the two endpoints in our previous study (Kang *et al.*, 2011). This difference was intriguing, given that the hepatic mRNA up-regulation was greater in ApoE$^{-/-}$ mice (~3.5-fold) compared with ~1.5- to 3.0-fold in wild-type mice. Although it is common to see no direct correlation between mRNA levels of the genes in certain tissue and their serum protein levels due to various technical and biological reasons, such as difference in sample preparation, different translational efficiency and dilution/degradation of the proteins during transport to the blood stream, in this case, there might be another important factor to consider - a technical issue with measuring SAP using ELISA in hyperlipidemic serum. It has been reported that low density lipoprotein (LDL) and very low density lipoprotein (VLDL) could selectively bind with CRP (de Beer *et al.*, 1982). Since SAP is known to resemble CRP structurally (Pepys *et al.*, 1979; Whitehead *et al.*, 1990), it is possible that SAP also bind with high levels of LDL and VLDL in ApoE$^{-/-}$ mouse serum and could not be detected by ELISA. Future studies using various detection methods would help to address this technical issue and better understand regulation of SAP in mice.

Another strength of this study is that we used occupationally realistic exposure concentrations - the highest exposure concentration we used (NH-1000: 673.6 μg Ni/m^3) was still below the OSHA PEL for nickel hydroxide, 1 mg Ni/m^3 (TOXNET Toxicology Data Network, 2003). Although nanoparticle-specific exposure guidelines are yet to be established and no actual workplace exposure data has been reported for nano-NH, this concentration is within the range of occupational exposure levels in various Ni industries (Sivulka *et al.*, 2007). Our result clearly demonstrated that nano-NH can induce acute phase response in mice at the exposure concentration below the current OSHA PEL, and this may imply a serious occupational health issue considering the growing interest in nano-NH in power/energy industry. Additional research is warranted to further evaluate potential health risks of nano-NH and establish nanomaterial-specific exposure guidelines in occupational and environmental settings.

Previously, we found significant increase in the hepatic *Sap* mRNA level after 1w exposure to nano-NH while other endpoints for systemic inflammation showed lack of response (Kang *et al.*, 2011). Here we report that SAP can be induced after only 1d exposure to nano-NH. These findings indicate

that SAP can be used as a marker for early detection of systemic inflammation in mice. In addition, both studies showed SAP induction after repeated exposures (3d, 1w, and 5 m), and it may suggest the potential use of SAP as an indicator of chronic inflammation in mice just like its human equivalent, CRP. Taken together, we believe that studying SAP will help better understand regulation of acute phase response following particle exposure, and its role in cardiovascular effects.

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