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Metal Ion Release of Manufactured Metal Oxide Nanoparticles Is Involved in the Allergic Response to Inhaled Ovalbumin in Mice

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Abstract

The aim of the present study was to establish the mechanism of the allergy aggravation effect. Our previous study showed that soluble ZnO nanoparticles caused allergy aggravation, but insoluble TiO_2 and SiO_2 nanoparticles did not induce an allergic response. Metal ion release is associated with the cytotoxicity of manufactured nanoparticles; however, the role of metal ion release in allergy aggravation remains to be elucidated. Therefore, we examined the allergy aggravation potential of several soluble manufactured nanoparticles (ZnO, CuO, NiO, MgO, and $CaCO_3$). These nanoparticles were administered to mouse lungs by pharyngeal aspiration and subsequently, the mice inhaled ovalbumin (OVA). We also compared the properties of soluble NiO nanoparticles with insoluble micro-scale NiO particles. NiO nanoparticles markedly increased the levels of OVA-specific immunoglobulin (Ig) E but micro-scale NiO particles did not. Among the nanoparticles (ZnO, CuO, MgO, and $CaCO_3$), ZnO induced increase of OVA-specific IgE level. CuO showed tendency to increase OVA-specific IgE; however, no significant difference was observed. Additionally, ZnO and NiO nanoparticles enhanced expression of a gene related to inflammation (Cxcl2), heavy metal detox (metallothionein 2), and oxidative stress (heme oxygenase-1). Gene expression of arginase1, which is enhanced by T helper 2 cytokine, was remarkably enhanced in mice administered ZnO and NiO particles. These effects were not observed in mice administered MgO and $CaCO_3$ nanoparticles. In conclusion, the solubility and type of metal ion released from the nanoparticles influence

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the allergy aggravation effect. The results showed that the release of Zn^{2+} and Ni^{2+} aggravated the allergic reaction.

Keywords

Nanoparticle, Inhalation, Allergy, Zinc Oxide, Nickel Oxide

1. Introduction

Nanoparticles are defined as particles whose diameter is 100 nm or less (ISO/TS 27687:2008). Currently, several nanoparticles are produced, particularly, metal oxide nanoparticles, such as TiO_2 and ZnO, which are widely used. They are used in the industry and for products such as catalysis, paint, cosmetics, and sunscreen. The manufactured nanoparticles contain both a harmful component and a harmless component. Since nanoparticles are defined by their diameter only, they can contain various chemical components. The toxic activity is dependent on the chemical components and properties. Generally, the toxicities of TiO_2 and CeO_2 nanoparticles are low [1]-[3]. On the contrary, ZnO, NiO, and CuO nanoparticles have demonstrated toxicity in cultured cells and animals [4]-[6]. Further, the physical and chemical properties of nanoparticles can influence the toxicity [7] [8]. In many cases, "toxicity" of manufactured nanoparticles is described as cytotoxicity (decrease of cell viability and cell membrane damage), induction of oxidative stress, induction of inflammation, and carcinogenicity. In addition to their direct toxicity effect, manufactured nanoparticles are also suggested to cause indirect toxicity. Namely, some manufactured nanoparticles have shown potential allergy aggravation effect. Pre-treatment of ZnO nanoparticles in mice by pharyngeal aspiration, and subsequent inhalation of ovo-albumin (OVA) markedly increased OVA specific immunoglobulin (Ig) E [9]. However, the allergy aggravation effect was not observed with TiO_2 and SiO_2 nanoparticles. In our previous study, we concluded that the allergy aggravation effect of ZnO nanoparticles was attributed to Zn^{2+} released from ZnO nanoparticles. Metal ion release is one of the features of metal oxide nanoparticles. NiO is classified as a water insoluble compound, however, some NiO nanoparticles have been shown to dissolve in cell culture medium [10]. In several cases, the released metal ion from nanoparticles is a major influence of their toxicity. NiO nanoparticles have demonstrated severe cytotoxicity on human lung carcinoma A549 cells and human keratinocyte HaCaT cells [10]. CuO nanoparticles and ZnO nanoparticles have also shown cytotoxic activity [7] [11]. In addition, these soluble nanoparticles have shown other toxic activity *in vivo*, such as induction of inflammation [12] [13]. These toxic effects of nanoparticles were suggested to be a direct effect. Despite evidence of the importance of metal ion release on the direct toxicity of nanoparticles, the indirect effect of released metal ions, such as the allergy aggravation effect remains to be elucidated. There is only one study that examined the role of ZnO nanoparticles on the allergic response [9]. Understanding the mechanism of the allergy aggravation effect is necessary for beneficial and effective use of manufactured nanoparticles. In the present study, we investigated the potential allergy aggravation of several soluble manufactured nanoparticles.

2. Materials and Methods

2.1. Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of University of Occupational and Environmental Health, Japan. The authorization reference number was AE11-008. Seven-week-old female C57BL/6N mice were obtained from Charles River Laboratories Japan, Inc. (Yokohama, Japan). Mice were fed a standard diet and were allowed to acclimatize to the environment (with a 12 h light/dark cycle) for 1 week before experimentation.

2.2. Nanoparticles and Chemicals

Four manufactured metal oxide nanoparticles (ZnO, CuO, NiO, and MgO) and $CaCO_3$ were evaluated. ZnO nanoparticles were purchased from Ishihara Sangyo Kaisha Ltd. (Osaka, Japan). CuO nanoparticles were purchased from CIK NanoTek Corporation (Tokyo, Japan). NiO (green) and MgO nanoparticles were purchased

from Nanostructured & Amorphous Materials, Inc. (Houston, TX). $CaCO_3$ nanoparticle (Hakuenka-O) was obtained from Shiraishi Kogyo Kaisha, Ltd. (Amagasaki, Japan). Fine (micro-scale) green NiO particles (Sumitomo Metal Mining Co., Ltd., Tokyo, Japan) were also examined. The physical properties of these particles, including the primary particle size, specific surface area, and purity have been previously reported (Horie *et al*., 2012c; Horie *et al*., 2009). The physical properties of these particles are shown in **Table 1**. For experiment 1, chicken egg albumin (OVA) was purchased from Seikagaku Corporation (Tokyo, Japan). For experiment 2, OVA was purchased from Kanto Chemical Co., Inc. (01103-31, Tokyo, Japan) because Seikagaku Corporation has stopped the production of OVA. Lipopolysaccharide (LPS) contamination of OVA was evaluated by an EndoLISA ELISA-based endotoxin quantification assay kit (Hyglos GmbH, Bernried am Starnberger See, Germany). LPS was not present in the OVA used in the present study.

2.3. Preparation of Nanoparticle Dispersion

Nanoparticle powder was added to phosphate buffered saline (PBS) at a concentration of 1 mg/ml. The nanoparticles were dispersed by sonication for 2 min by using Bioruptor UCD-250 (Tosho Denki, Samukawa, Japan). Nanoparticle dispersions were immediately used for aspiration after preparation. Although the nanoparticles formed aggregated in the PBS, there were no large aggregates that blocked the syringe during aspiration. The concentration of released metals in the dispersion was measured after removing the particles by ultrafiltration. The concentration of Ni^{2+} in the dispersion was determined by 5-Br-PSAA (Dojindo Laboratories, Kumamoto, Japan). The concentration of Zn^{2+} was determined by 5-Br-PAPS (Dojindo). Concentrations of Cu^{2+} and Mg^{2+} were determined by Metallo Assay Copper LS and Metallo Assay Mg LS (Metallogenics Co. Ltd., Chiba, Japan), respectively. Metallo Assay Copper LS and Metallo Assay Mg LSwere based on the DiBr-PAESA method and XB-I method, respectively. The concentration of Ca^{2+} was determined by the MXB method using a Calcium-E test (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

2.4. Exposure Examination

An outline of the exposure protocol is shown in **Figure 1**. The mice were divided into eight groups, with five mice in each group (n = 5). Fifty microliters of nanoparticle dispersion (1.0 mg/mL) was administered to the lungs by pharyngeal aspiration under intraperitoneal anesthesia of pentobarbital sodium salt (8.1 mg/mL) (Somnopentyl; Kyoritsu Seiyaku Corporation, Tokyo, Japan. Eight times dilution with PBS). Fifty microliter of bovine serum albumin (BSA) solution (control) was administered to the lungs by pharyngeal aspiration. Then, OVA solution was administered by nasal exposure using a nebulizer (Porta-Neb: Medic-Aid Ltd., Bognor Regis, UK). OVA solution was nebulized by a nebulizer and a mist of the OVA was homogenized. Mice inhaled the mist by nasal exposure. Nasal exposure was conducted four times every other day (10 min/day). After a break of 1 week, nasal exposure was conducted again four times every other day. In total, the mice were exposed to OVA eight times. The day after the final exposure, mice were anesthetized with intraperitoneal injection of pentobarbital sodium salt (32.4 mg/mL). At autopsy, blood was obtained from the heart and the lung was removed.

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

Serum was prepared by centrifugation of whole blood at 5000 rpm, for 5 min. Serum concentration of total IgE,

Table 1. Properties of nanoparticles used in this study.

Material	Abbreviation in this study	Primary particle size (nm)	Specific surface area (m^2/g)
NiO	nNiO	<100	74.9
NiO	fNiO	600 - 1400	3.44
ZnO	ZnO	21	31.5
CuO	CuO	48	12.8
MgO	MgO	20	44.2
$CaCO_3$	$CaCO_3$	30	ND

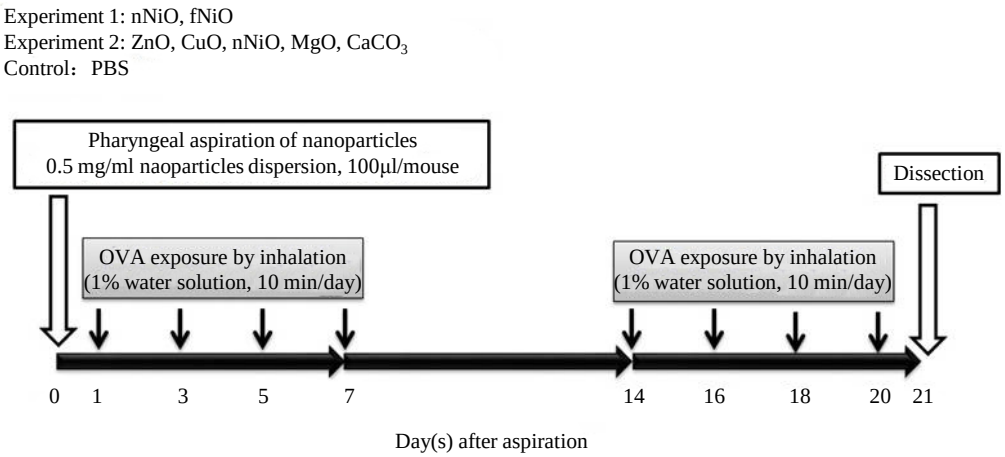

Figure 1. Experimental design of nanoparticle aspiration and OVA inhalation.

total IgG, OVA specific IgE, and OVA-specific IgG_1 were determined by ELISA. Total IgE concentration was determined by mouse IgE EIA kit (Yamasa Corporation, Choshi, Japan). Total IgG was determined by mouse IgG total ELISA Ready-SET-Go! (eBioscience, Inc., San Diego, CA, USA). OVA specific IgE was determined by DS mouse IgE ELISA (OVA) (DS Phama Biomedical Co., Ltd., Osaka, Japan). OVA-specific IgG_1 was determined by a mouse Anti-OVA-IgG_1 ELISA Kit (Shibayagi Co., Ltd., Shibukawa, Japan). ELISAs were performed according to the manufacturers' protocol.

2.6. Real Time-Polymerase Chain Reaction (Real Time-PCR)

The lungs were soaked in RNAlater RNA stabilization reagent (Qiagen, Hilden, Germany) immediately. Lung tissue was homogenized by TissueRuptor (Qiagen) and total RNA was prepared by RNeasy Mini kit (Qiagen). Real time-PCR was conducted using step one real time-PCR system (Applied Biosystems), and PCR amplification of lung tissue was analyzed by TaqMan® gene expression assays (Life Technologies Corp.), with the *β*-actin gene of rats as the endogenous control. The code of the probe for TaqMan® gene expression assays for *β*-actin, heme oxygenase-1 (HO-1), metallothionein 2 (MT2), tumor necrosis factor (TNF-*α*), chemokine (C-X-C motif) ligand 2 (Cxcl2), interleukin-1 beta (IL-1*β*), nitric oxide synthase 2 (NOS2), and arginase (Arg1) were Mm00607939_s1, Mm00516005_m1, Mm00496660_g1, Mm00443260_g1, Mm00436450_m1, Mm00434228_m1, Mm03294838_g1 and Mm00475988_m1, respectively.

2.7. Statistical Analysis

The data are expressed as the mean ± S.E. Statistical analyses were performed by analysis of variance (ANOVA) with Dunnett tests. Significant differences against PBS group were indicated in each figure.

3. Results

3.1. Effect of Micro-Scale Particle and Nanoparticle by NiO Aspiration and Subsequent OVA Inhalation on the Blood Concentration of IgE and IgG, and Gene Expression in the Lungs (Experiment 1)

NiO nanoparticles (nNiO) and micro-scale NiO particles (fNiO) were evaluated for their ability to aggravate allergy. NiO suspensions (50 µg/mouse) were injected into the lungs of mice by pharyngeal aspiration, and OVA (1%) was subsequently inhaled eight times (**Figure 1**). The same procedure was performed with PBS, as the negative control. Although the mice did not die in the PBS and fNiO groups, 4 mice were found dead in the nNiO group (n = 5), 3 days after first OVA inhalation. Therefore, the value of nNiO in **Figure 2** and **Figure 3** was obtained from one mouse only. Statistical analysis of the nNiO group was not performed. Compared with the PBS group, the concentration of total blood IgE, OVA-specific IgE, and OVA-specific IgG_1 increased in the nNiO group after OVA exposure (**Figure 2**). Gene expression in lung tissues after OVA exposure was examined

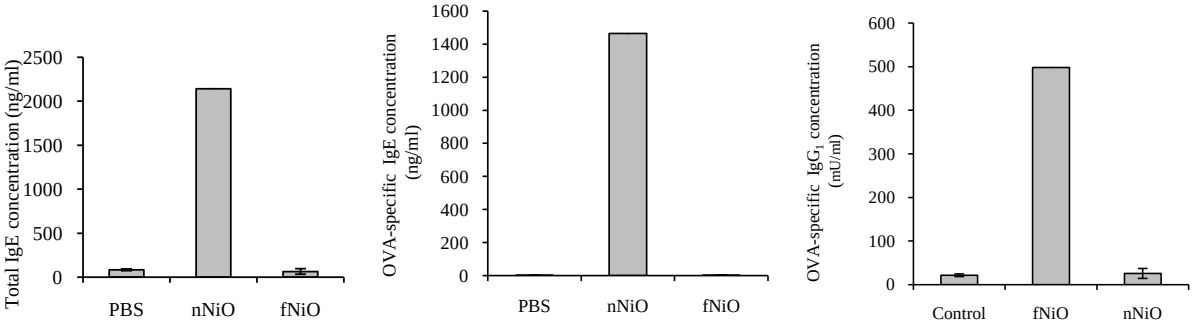

Figure 2. Gene expression in the lung tissue of NiO particle-aspirated mice following OVA exposure (experiment 1). nNiO, fNiO and PBS were administered to mouse lungs by pharyngeal aspiration. Subse quently, the mice were exposed to OVA. The experimental design is shown in **Figure 1**. nNiO group could not carry out statistics analysis because 4 mice died during inhalation of OVA. There was no significant difference between the PBS and the fNiO groups.

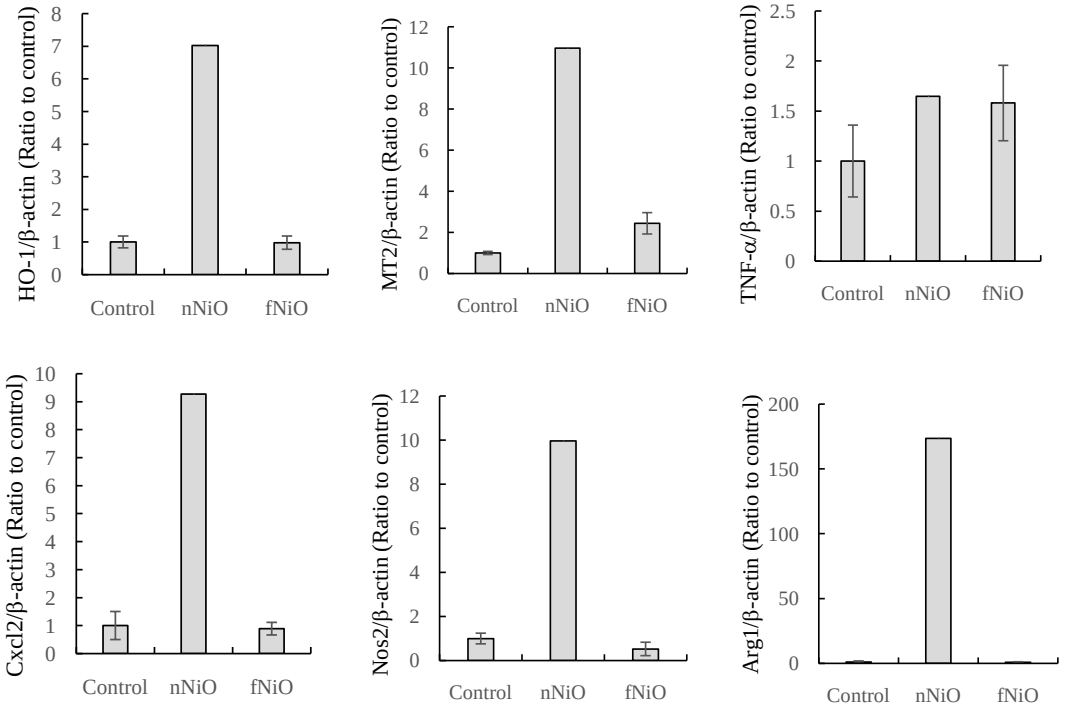

Figure 3. Gene expression in the lung tissue of NiO particle-aspirated mice following OVA exposure (experiment 1). nNiO, fNiO and PBS were administered to mouse lungs by pharyngeal aspiration. Subsequently, the mice were exposed to OVA. The experimental design is shown in **Figure 1**. Gene expression was determined by real-time PCR. nNiO group could not carry out statistics analysis because 4 mice died during inhalation of OVA. There was no significant difference between the PBS and the fNiO groups.

(**Figure 3**). Gene expression of oxidative stress response protein HO-1 was higher in the nNiO group than the other groups. In addition, the expression of Cxcl2, a chemokine for neutrophils, increased in the nNiO group. The nNiO group showed remarkable gene expression of arginase, which is expressed in M2 macrophages. Gene expression of metal response protein MT2 and NOS2 were also higher in the nNiO group than the other groups.

3.2. Effect of Various Metal Oxide Nanoparticles by Aspiration and Subsequent OVA Inhalation on the Blood Concentration of IgE and IgG (Experiment 2)

Four different manufactured metal oxide nanoparticles (ZnO, CuO, NiO, and MgO) and $CaCO_3$ nanoparticles were evaluated for their ability to aggravate allergy. Pharyngeal aspiration of PBS was considered as the nega-

tive control, and the negative control group did not differ significantly to the untreated animals that did not undergo pharyngeal aspiration and OVA exposure. After pharyngeal aspiration of nanoparticles, mice inhaled OVA eight times in total (**Figure 1**). Although the mice did not die in the PBS and fNiO groups, 3 mice were found dead in the nNiO group (n = 5) 3 days after the first OVA inhalation. Therefore, the values of nNiO in **Figure 2** and **Figure 3** were obtained from 2 mice only. Statistical analysis of the nNiO group was not performed. There were no significant differences observed in body weight between the PBS group and the nanoparticles groups except the NiO group during OVA exposure (**Figure 4**). Although statistical analysis was not carried out, the body weight of the NiO group showed a tendency to decrease 7 days after aspiration. The ZnO groups showed an increase in blood concentration of IgE and OVA-specific IgE (**Figure 5**). In the ZnO group, OVA-specific IgE level was approximately 358 times higher than that in the PBS group. However, increase of OVA-specific IgE was not observed in the CuO, MgO, and$CaCO_3$ groups. The NiO group (n = 2) showed tendency to increase the blood concentration of IgE and OVA-specific IgE. In the NiO group, the OVA-specific IgE level was approximately 168 times higher than that in the PBS group. The blood concentration of total IgG concentration was not affected by pharyngeal aspiration of metal oxide and $CaCO_3$ nanoparticles. The concentration of OVA-specific IgG_1 increased in both the ZnO and CuO groups. The OVA-specific IgG_1 levels in the ZnO and CuO groups were approximately 27 and 26 times higher than those in the PBS group, respectively. NiO group (n = 2) also showed tendency to increase the blood concentration of IgG_1. In the NiO group, the OVA-specific IgG_1 level was approximately 35 times higher than that in the PBS group.

3.3. Effect of Nanoparticle Aspiration and Subsequent OVA Inhalation on Gene Expression in the Lungs (Experiment 1)

We evaluated the relationship between oxidative stress, inflammation, and allergic activity. ZnO and NiO nanoparticles induced an increase of intracellular ROS level and HO-1 expression [4] [12]. Intratracheal instillation of some nanoparticles caused pulmonary inflammation [14]. Further, the suppression of inducible nitric oxide synthase (iNOS; NOS2) has been suggested to be involved in the allergic response of diesel exhaust particles (DEP) [15]. After pharyngeal aspiration of nanoparticles and inhalation of OVA, lung tissues were obtained and the gene expressions of stress response proteins, cytokines, and differentiation markers were examined (**Figure 6**). Expression of genes encoding the oxidative stress response protein HO-1 in the ZnO and NiO (n = 2) groups were 2.2 and 3.3 times higher than that in the PBS group, respectively. The expression of Cxcl2 in the ZnO and NiO (n = 2) groups were 3.5 and 4.7 times higher than that in the PBS group, respectively. The expression of the metal response protein MT-2 gene in the ZnO group was two times higher than that in the PBS group. Gene expression of cytokines, TNF-a, and IL-1b was not affected. NOS2 and Arg1 genes increased in the ZnO and NiO (n = 2) groups. Particularly, the expression of Arg1 in the ZnO and NiO groups were 29 and 52 times higher than that in the PBS group, respectively.

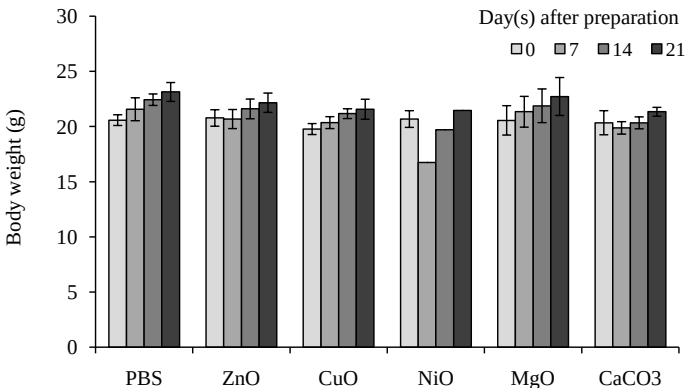

Figure 4. Body weight of mice (experiment 2). Body weight was measured at 0, 7, 14, and 21 day (s) after pharyngeal aspiration of nanoparticles. The values represent the mean ± SE. There were no significant differences among the groups. NiO group could not carry out statistics analysis because 3 mice died during inhalation of OVA.

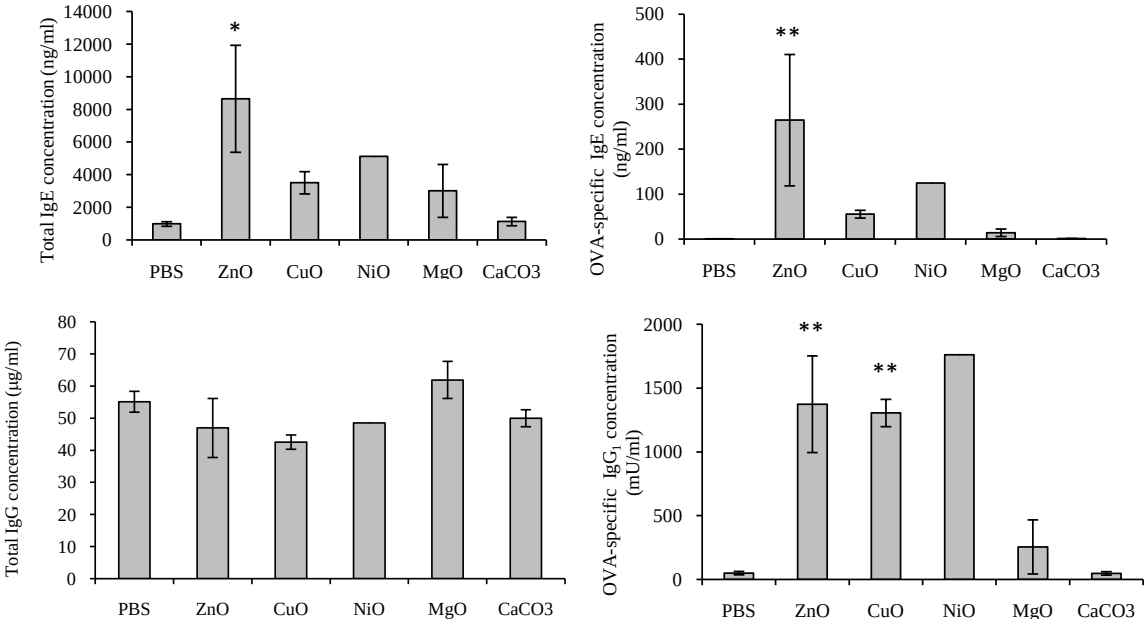

Figure 5. Concentration of total IgE, OVA-specific IgE, total IgG and OVA-specific IgG1 in the blood of various nanoparticles-aspirated mice following OVA exposure (experiment 2). ZnO, CuO, NiO, MgO, CaCO3, and PBS were administered to mouse lung by pharyngeal aspiration. The mice were then exposed to OVA. The experimental design is shown in **Figure 1**. The values represent the mean ± SE. $^{**}p < 0.01$ vs. PBS group, Dunnett, ANOVA. NiO group could not carry out statistics analysis because 3 mice died during inhalation of OVA.

4. Discussion

In the present study, pharyngeal aspiration of NiO nanoparticles strongly aggravated the allergic reaction to inhaled ovalbumin in mice. Micro-scale NiO particles did not induce aggravation of the allergic reaction. Micro-scale NiO particles are generally, classified as a water-insoluble compound, but NiO nanoparticles had high solubility in biological fluids, such as culture medium [10]. Enhancement of the MT2 gene expression in the lungs of mice administered NiO nanoparticles, indicated dissolution of NiO nanoparticles in the lungs on the 21st day after injection. Additionally, gene expressions of HO-1 and Cxcl2 were also enhanced in the lungs of mice administered NiO nanoparticles. These observations suggest that Ni^{2+} released from NiO nanoparticles induced oxidative stress and inflammation. Moreover, gene expressions of Nos2 and Arg1 in the lungs were enhanced by NiO nanoparticles. The expression of NOS2 (also known as iNOS) was induced by T helper (Th) 1 cytokine and Arg1 was enhanced by Th2 cytokine [16] [17]. Expression of NOS2 was suppressed by Th2 cytokine, and the expression of Arg1 was induced by Th2 cytokine [18]-[20]. The regulation of Th1/Th2 levels is important in allergic reactions. The gene expression of NOS2 in the NiO administered mice was approximately 10 times higher than that of the control mice. On the other hand, the gene expression of Arg1 in the NiO nanoparticles injection mice was approximately 170 times higher than that of the control mice. However, micro-scale NiO particles that did not release Ni^{2+}, did not enhance the gene expression of Nos2 nor Arg1. These results suggest that Ni^{2+} released from NiO nanoparticles is an important factor of the allergy aggravation reaction. Moreover, the allergy aggravation effects of various metal oxide nanoparticles (ZnO, CuO, and MgO) and $CaCO_3$were examined. All nanoparticles employed in this study dissolved in culture medium [7] [21]. Among the manufactured nanoparticles, ZnO and NiO showed the strongest allergy aggravation effect. CuO nanoparticles also showed tendency to aggravate allergic reaction. In contrast, MgO and $CaCO_3$ nanoparticles demonstrated no allergy aggravation effects. Pharyngeal aspiration of MgO and $CaCO_3$ nanoparticles did not affect the gene expression. When ZnO nanoparticles showed aggravation effect, the gene expressions of HO-1, Cxcl2, and MT2 were enhanced. The gene expressions of NOS2 and Arg1 of mice administered ZnO nanoparticles were approximately 1.5 and 29 times higher than that of the control mice, respectively. This suggests that Zn^{2+} released from ZnO nanoparticles is an important factor of the allergy aggravation reaction, as well as NiO nanoparticles. However, our previous study showed that pharyngeal aspiration of soluble zinc compound, $ZnCl_2$, did not cause

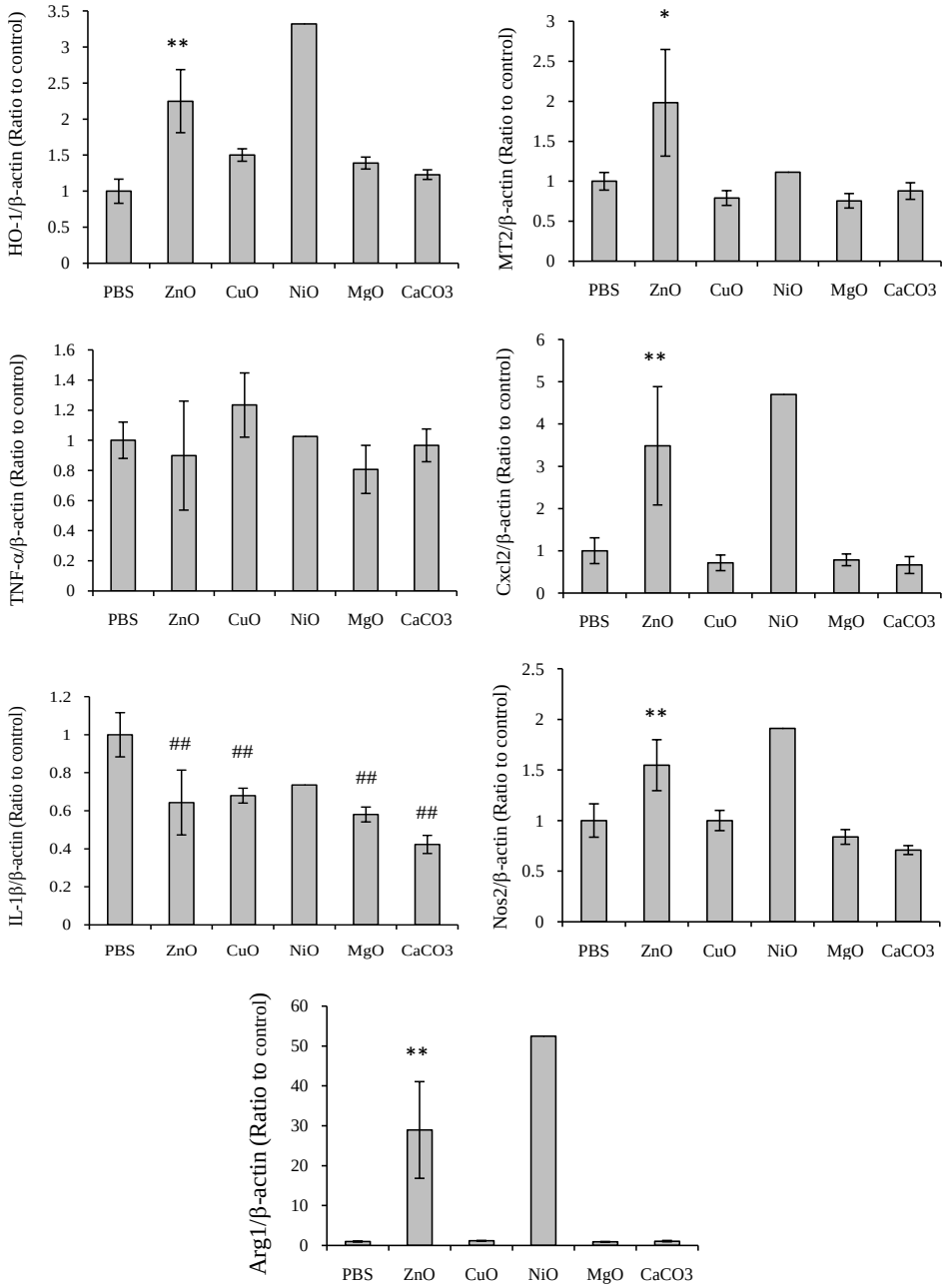

Figure 6. Gene expression in the lung tissue of zinc compound-aspirated mice following OVA exposure (experiment 2). ZnO, CuO, NiO, MgO, CaCO3, and PBS were administered to mouse lungs by pharyngeal aspiration. Subsequently, the mice were exposed to OVA. The experimental design is shown in **Figure 1**. Gene expression was determined by real-time PCR. The values represent the mean ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. PBS group, Dunnett, ANOVA. NiO group could not carry out statistics analysis because 3 mice died during inhalation of OVA.

an allergy aggravation effect [9]. Injected $ZnCl_2$ may be cleared from the lungs rapidly after administration. On the other hand, NiO and ZnO nanoparticles might stay in the lungs and continue to release Ni^{2+} and Zn^{2+}. Further, nanoparticles are taken up into lung epithelial cells [10] [22]. The gene expression of MT2 in the lungs was still enhanced 21 days after administration in mice administered NiO and ZnO. Therefore, the shape and solubility influence the allergy aggravation effects caused by metal oxide nanoparticles. The allergic reaction was not aggravated by metal ion alone. Even if particle size is nano-scale, metal-oxide nanoparticles that does not dis-

solve, could not aggravate the allergic response. In conclusion, some metal oxide nanoparticles have allergy aggravation ability. Solubility and the type of metal ion released from the nanoparticles influence the allergy aggravation effect. The release of Zn^{2+} and Ni^{2+} aggravated the allergy reaction but the release of Mg^{2+} and Ca^{2+} did not aggravate the allergy reaction. There is possibility that some metal oxide nanoparticles aggravate allergy reaction such as asthma and atopic dermatitis. In order to prevent allergy aggravation, prevention of nanoparticles inhalation is important, particularly, about workers in nanoparticle manufacturing plant.

5. Conclusion

Metal ion release from metal oxide nanoparticles is one of the important factors of their allergy aggravation effect.

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