

Metal Allergens Induce Nitric Oxide Production by Mouse Dermal Fibroblasts via the Hypoxia-Inducible Factor-2 α -Dependent Pathway

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Nickel (Ni) has been shown to be one of the most frequent metal allergens. We have already reported a murine metal allergy model with pathogen-associated molecular patterns (PAMPs) as adjuvants. Interleukin (IL)-1 β plays a critical role in our mouse model. Because nonimmune cells, including fibroblasts, play important roles in local allergic inflammation, we investigated whether Ni induces inflammatory responses in mouse dermal fibroblasts (MDF). We also analyzed the synergistic effects between Ni, PAMPs, and IL-1 β . MDF stimulated with Ni produced a significantly higher amount of nitric oxide (NO) in a dose-dependent manner. NO production was augmented by costimulation with IL-1 β but not with PAMPs. On the other hand, IL-1 β or PAMPs induced a significantly higher amount of IL-6 production by MDF, but no augmentation was detected in the presence of Ni. A specific inhibitor for inducible nitric oxide synthase (iNOS) inhibited Ni-induced NO production. iNOS mRNA expression was significantly higher in MDF stimulated with Ni, IL-1 β , or both. A specific inhibitor for hypoxia-inducible factor (HIF)-2 α , but not HIF-1 α , inhibited NO production. Another frequent metal allergen, cobalt, also induced iNOS expression and NO production by MDF via the HIF-2 α -dependent pathway. The inhibitor for iNOS augmented ear swelling in Ni allergy mouse model. On the other hand, HIF-2 α inhibitor attenuates allergic inflammation. These results indicate that metal allergens induce NO production in MDF via the HIF-2 α -dependent pathway and IL-1 β augments NO production, which suggests that the NO induced by metal allergens plays a pathological role in metal allergies.

Key Words: Ni; IL-1 β ; HIF; NO; dermal fibroblasts.

Metal allergies have been classified as a type IV allergy mediated by helper 1 T lymphocytes and generally induce contact hypersensitivity (CHS) (Thyssen and Menné, 2010). Nickel (Ni) has been most frequently detected among various metals as a metal allergen (Thyssen and Menné, 2010). Ni ions and insoluble particles can be taken into cells by ion channels or active phagocytosis, respectively, and induce many

cellular events (Kasprzak *et al.*, 2003). Ni has been shown to induce the expression of chemokine receptors and production of proinflammatory cytokines in human dendritic cells (DCs) (Antonios *et al.*, 2010; Boislève *et al.*, 2004). It has recently been reported that human toll-like receptor (TLR) 4, but not the mouse homologue, is directly activated by Ni and cobalt (Co) (Raghavan *et al.*, 2012; Schmidt *et al.*, 2010).

Ni exhibits hypoxia-mimicking activity. Ni inhibits prolyl hydroxylase (PHD), which catalyzes prolyl hydroxylation of hypoxia-inducible factor (HIF)- α (Ke and Costa, 2006). HIF is a heterodimeric transcriptional factor consisting of α and β subunits and binds to hypoxia-responsive element in the promoter region. There are three known active α subunits, HIF-1 α , 2 α , and 3 α , and the single β subunit HIF-1 β . Under normoxic conditions, HIF- α is hydroxylated by PHDs and subsequently degraded by proteasome. Under hypoxic conditions, PHDs are inhibited and HIF- α escapes degradation. The stabilized α subunit translocates to the nucleus and associates with the β subunit. Ni induces proangiogenic mediators and proinflammatory cytokines via HIF-1 α -dependent pathway (Brant and Fabisiak, 2009).

We previously reported an effective Ni allergy mouse model that uses lipopolysaccharide (LPS) as an adjuvant (Sato *et al.*, 2007). In this model, interleukin (IL)-1 is indispensable. Moreover, LPS augmented the allergy to Ni, not only in the sensitization but also in the elicitation steps (Kinbara *et al.*, 2011b). These findings indicated that IL-1 and pathogen-associated molecular patterns (PAMPs) interact with Ni to elicit both immunological and inflammatory responses.

Nonimmune cells in local tissues, such as fibroblasts, also play indispensable roles in allergic inflammation. Dermal fibroblasts stimulated by tumor necrosis factor- α and IL-1 β have been shown to activate matrix metalloproteinase-9 production and the migration of DCs (Saalbach *et al.*, 2010). The activated dermal fibroblasts produce prostaglandin E₂, which induces Th17-related cytokine IL-23 production from DCs (Schirmer

et al., 2010). Therefore, it is important to investigate the effects of metal allergens on dermal fibroblasts in order to clarify the pathological mechanism of metal allergies.

Nitric oxide (NO) is a multifunctional gas mediator that is involved in a variety of events (Bogdan, 2001). NO is synthesized by the intracellular enzyme NO synthetase (NOS), which has three isoforms, neural NOS (nNOS, NOS1) and endothelial NOS (eNOS, NOS3), which are constitutive, and inducible NOS (iNOS, NOS2) (Bogdan, 2001). iNOS is induced in fibroblasts stimulated by various inflammatory agents including IL-1 and LPS (Jorens *et al.*, 1992; Wang *et al.*, 1996). Ni was shown to induce iNOS expression *in vivo* and *in vitro* (Cruz *et al.*, 2004; Gupta *et al.*, 2000). HIF also regulates iNOS expression (Melillo *et al.*, 1995). However, to the best of our knowledge, no study has shown the direct involvement of HIF with the induction of iNOS by Ni.

NO shows dual effects in the pathogenesis of CHS (Ross and Reske-Kunz, 2001). In early phase, concentration of NO is relatively low, and NO shows proinflammatory effects, such as tissues damages, vasodilation, and attraction of neutrophils. Langerhans cells, a DC subtype localized in epithelium, and keratinocytes express iNOS by stimulation with contact allergens (Ross and Reske-Kunz, 2001). In late phase, activated DC and macrophages express iNOS and the concentrations of NO in local inflammatory sites increase. This high NO level shows anti-inflammatory effects, such as apoptosis of effector cells and downregulation of the expression of cell adhesion molecules. Because of the dual effects of NO in CHS, contradictory results were reported. NO-releasing cream on normal skin has been shown to induce significant increases in inflammatory conditions (Ormerod *et al.*, 1999). NOS inhibitors suppressed CHS response when the inhibitor was applied at elicitation phase (Ross *et al.*, 1998). On the other hand, NO-releasing hydrocortisone shows greater protective effects than its parental compound (Hyun *et al.*, 2004). iNOS downmodulates CHS response by suppressing DC migration and survival (Sugita *et al.*, 2010).

In this study, we analyzed NO production by mouse dermal fibroblasts (MDF) stimulated with Ni, PAMPs, and the proinflammatory cytokine IL-1 β . We also analyzed the HIF-dependent signals involved in Ni-induced NO production. Finally, we analyzed *in vivo* effects of iNOS and HIF inhibitors on the Ni allergy mouse model.

MATERIALS AND METHODS

Reagents. HIF-1 α and HIF-2 α translation inhibitors were purchased from Merck Millipore (Billerica, MA). LPS from *Escherichia coli* O55:B5, prepared by Westphal's method, was purchased from Difco Laboratories (Detroit, MI). Pam₃Cys-Ser-(Lys)₄ (Pam₃CSK₄) was purchased from Merck Millipore. Recombinant mouse IL-1 β was purchased from BioLegend (San Diego, CA). All other reagents were purchased from Wako pure chemicals (Osaka, Japan), unless otherwise indicated.

Cell culture. MDF were isolated from male BALB/cA mice. After shaving the back, skin tissue was removed and rinsed twice with PBS containing

antibiotics (Antibiotic-Antimycotic, Life Technologies, Carlsbad, CA). The specimen was cut into 3–5 mm square pieces and placed in a plastic dish (10 cm diameter), with the dermal side facing the dish. To fix the specimen on the dish, it was incubated at 37°C for 20 min without medium and then cultured with 10 ml of culture medium (Dulbecco modified minimum essential medium containing 10% heat-inactivated fetal bovine serum [FBS] and antibiotics). Fibroblasts grew after 10–20 days of culture. The culture medium was changed every 2–3 days, and cells were passaged at 70–80% confluence using 0.25% Trypsin and 1 mM EDTA solution. Cells at passage 5–7 were used for experiments. For *in vitro* stimulation, cells were seeded in a 24-well culture plate at 2×10^4 per milliliter per well. After 2 days of culture, the culture supernatant was removed and cells were cultured with the indicated stimuli at 500 μ l final volume. For Western blotting, cells were seeded in a 10-cm-diameter dish at 4×10^5 /10 ml/dish. The culture supernatant was removed after 2 days of culture, and cells were cultured with the indicated stimuli at a final volume of 10 ml.

Cell proliferation and cytotoxicity assay. Cell proliferation was measured with a MTT assay (Cell Counting Kit-8, DOJINDO, Kumamoto, Japan). For the quantification of cytotoxicity, lactate dehydrogenase (LDH) activity in the culture supernatant was measured with a cytotoxicity detection kit (Roche Diagnostic, Indianapolis, IN).

Nitrite assay. Nitrite (NO₂⁻) concentrations were measured using Griess reagent (1% sulfanilamide, 0.1% n-(1-naphthyl)-ethylenediamine, 2.5% phosphoric acid) with NaNO₂ as the standard. The detection limit of the assay was higher than 2 μ M nitrite. To analyze significance, ND (not detected) was considered as 2 μ M.

Measurement of IL-6 in culture supernatants. The amount of IL-6 in the culture supernatant was measured with a commercial kit (mouse IL-6 ELISA max standard set, BioLegend).

Quantitative RT-PCR. Cells were lysed in 1 ml Isogen (Nippon Gene, Toyama, Japan), and total RNA was extracted as described in the instruction manual. Five hundred nanograms of total RNA was applied to cDNA synthesis using the Transcriptor First strand cDNA synthesis kit (Roche Diagnostic). Real-time PCR was performed with LightCycler Fast Start DNA Master SYBR Green I and LightCycler 1.5 Systems (Roche Diagnostic). The primers used for PCR were as follows: iNOS, forward 5'-GCAAACATCAGATTCAGATCCC-3' and reverse 5'-TCAGCCTCATGGTAAACACG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-CTTTGTCAAGCTCATTCCTGG-3' and reverse 5'-TCTTGCTCAGTGTCTTGC-3'. PCR conditions were 35 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. The product sizes for iNOS and GAPDH were 150 and 133 bp, respectively. The specificity of the PCR was confirmed by the product sizes with gel running and melting curve analysis for each data point. mRNA expression levels were expressed as relative units after normalization by the GAPDH level.

Western blotting. Cells were lysed in RIPA buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO). After centrifugation, the protein concentrations of cell lysates were determined by measuring absorbance at 280 nm. Cell lysates were separated by SDS-PAGE under reducing conditions. After electrophoresis, gel proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). The blot was blocked for 1 h with 3% (wt/vol) skim milk and 0.05% Tween 20 in PBS and incubated with the first Ab (anti-iNOS, Abcam, Cambridge, UK) followed by the horseradish peroxidase-conjugated second Ab (Thermo Scientific, Waltham, MA). After washing, the blot was analyzed with the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and LAS 4000 mini image analyzer (Fuji Film, Tokyo, Japan). After stripping with 2 M Glycine-HCl (pH 2.8) at room temperature for 1 h, the membrane was incubated again with anti- β -actin (BioLegend) and an appropriate second Ab to confirm equal loading of the protein.

Ni allergy mouse model. Ni allergy mouse model was constructed as previously reported (Sato *et al.*, 2007). Briefly, mice (female BALB/cA) were

sensitized with an ip injection of 250 μ l of solution containing 0.5mM NiCl₂ and 0.5 μ g/ml LPS. Ten days after sensitization, the mice were challenged with 20 μ l of 1mM NiCl₂ by an intradermal injection into the both pinnae. Ear swelling was measured at the indicated times using Peacock dial thickness gouge.

Statistical analysis. Experiment values were given as the mean $\pm$ SD of a single experiment performed in triplicate. All of the experiments in this study were performed at least thrice to confirm the reproducibility of the results. The data shown are representative results. Statistical analysis was performed with the unpaired *t*-test between two groups, or a one-way ANOVA using Bonferroni's (to compare all groups) or Dunnett's (to compare between the control and others) method between three or more groups, and *p* < 0.05 was considered significant.

RESULTS

Cytotoxic Effects of Ni²⁺ on MDF

At first, we analyzed the cytotoxic effects of NiCl₂ (Ni²⁺) on MDF. As shown in Figure 1A, MDF proliferation was inhibited in a Ni²⁺ dose-dependent manner. In the presence of 100 μ M Ni²⁺, MDF proliferation after 4 days of culture was approximately 65% of the medium (0 μ M of Ni²⁺). To confirm that the lower cell proliferation is cytotoxic but not cytostatic effects, we also measured LDH activity in the culture supernatants as an indicator of cytoplasmic leakage. Because FBS shows high LDH activity, we used culture medium with 1% FBS to reduce background levels in the measurements of LDH activity. In the presence of 50 and 100 μ M Ni²⁺, approximately 40% cytotoxicity was measured after 4 days of culture (Fig. 1B).

These results indicated that a higher concentration of Ni²⁺ had cytotoxic effects on MDF.

NO Production Induced By Ni²⁺ and Augmentation By IL-1 β

Next, we measured nitrite concentrations in the culture supernatants as an indicator of NO production. As shown in Figure 2A, nitrite concentrations in the culture supernatants of MDF stimulated with Ni²⁺ for 4 days increased in a Ni²⁺ dose-dependent manner. The concentration of nitrite with 100 μ M Ni²⁺ was approximately 20 μ M, which was significantly higher than that of the control (0 μ M of Ni²⁺). A significant amount of nitrite was detected after 3 days of culture and increased in a time-dependent manner up to 4 days of culture (Fig. 2B). Moreover, another Ni salt, NiSO₄, but not another chloride, CaCl₂, also induced NO production by MDF (data not shown). To exclude the possibility of cytoplasmic leakage, we measured nitrite concentrations in whole cell lysates. However, no nitrite was detected even in whole cell lysates prepared from MDF stimulated with IL-1 β (data not shown). We also measured NO production in MDF stimulated with other chemical irritants, such as dimethyl sulfoxide, HCl, NaOH, SDS, and Triton X-100. However, no nitrite was detected in culture supernatants of MDF stimulated with chemical irritants (data not shown).

We previously reported that PAMPs were effective adjuvants for a Ni allergy and that IL-1 played an important role in the pathogenesis of a Ni allergy (Sato *et al.*, 2007). We then analyzed

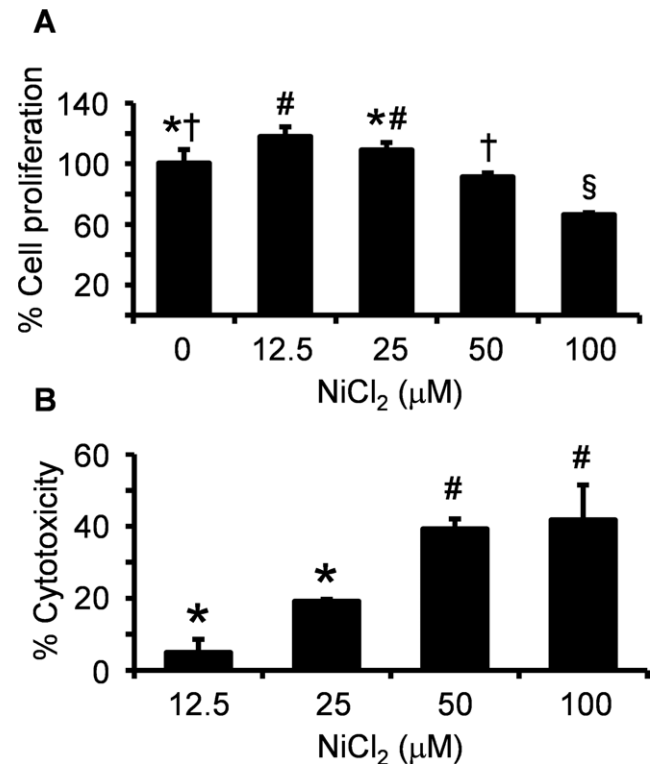


FIG. 1. Cytotoxic effects of Ni²⁺ on MDF. Cells were cultured in medium containing 10% (A) or 1% (B) FBS with the indicated amount of NiCl₂ at a final volume of 500 μ l for 4 days. (A) Cell proliferation was measured by a MTT assay. The results were expressed as % cell proliferation after being normalized to the values with 0 μ M NiCl₂. (B) Cytotoxicity was determined by measuring LDH activity in culture supernatants. The results were expressed as % cytotoxicity after being normalized to the values with 0 μ M NiCl₂ as 0% and those with 2% Triton X-100 as 100%. The results were expressed as the mean $\pm$ SD of a single experiment performed in triplicate. Means without a common symbol differ, *p* < 0.05.

the synergistic effects of PAMPs, such as LPS (a TLR4 ligand), Pam₃CSK₄ (a TLR2 and 1 ligand), and IL-1 β to Ni²⁺-induced NO production. As shown in Figure 3A, only a traceable amount of nitrite was detected in the culture supernatant of MDF stimulated with IL-1 β alone at 10 and 100 ng/ml. In the case of costimulation with Ni²⁺ and IL-1 β , nitrite concentrations were significantly higher than those with Ni²⁺ alone. On the other hand, LPS and Pam₃CSK₄ alone did not induce NO production, and only a slight augmentation was detected when MDF were costimulated with higher concentrations (100 ng/ml) of LPS or Pam₃CSK₄ and Ni²⁺ (Fig. 3A). We also analyzed IL-6 production. Figure 3B shows that LPS, Pam₃CSK₄, and IL-1 β alone induced IL-6 production in MDF. Higher concentrations (100 ng/ml) of LPS and Pam₃CSK₄ induced approximately 60 ng/ml of IL-6. On the other hand, IL-1 β induced a higher amount of IL-6 (approximately 130 ng/ml) even at a lower concentration (10 ng/ml). Regardless of stimulation, IL-6 production decreased significantly in the presence of Ni²⁺, which may have been caused by the cytotoxic effects of Ni²⁺.

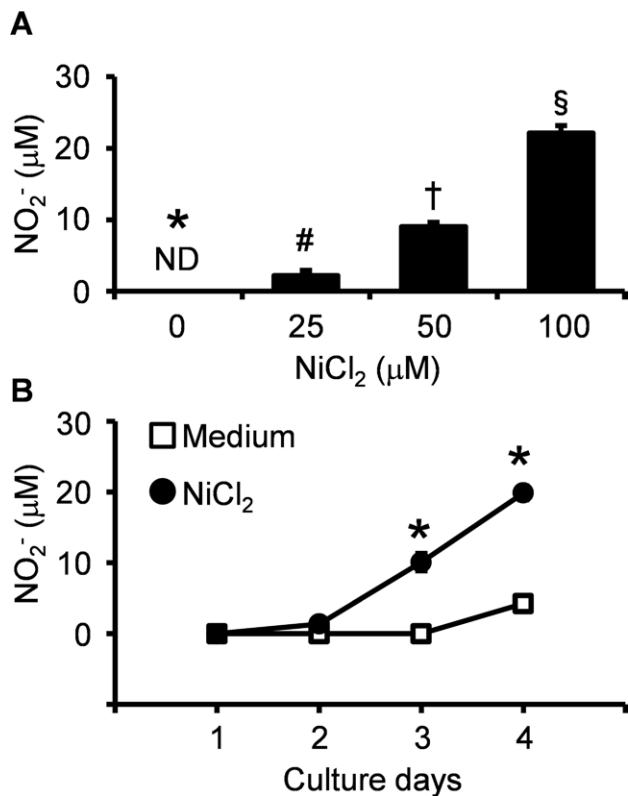


FIG. 2. NO production by MDF stimulated with Ni²⁺. Cells were cultured in medium containing 10% FBS with the indicated amount of NiCl₂ at a final volume of 500 μl. Nitrite concentrations were measured with Griess reagent. (A) Cells were stimulated for 4 days. ND, not detected. Means without a common symbol differ, $p < 0.05$. (B) Cells were stimulated with 100 μM of NiCl₂ for the indicated period. * $p < 0.05$, significantly different from the medium. The results were expressed as the mean $\pm$ SD of a single experiment performed in triplicate.

These results indicated that Ni²⁺ induced NO production in MDF in a dose- and time-dependent manner and IL-1 β augmented it.

iNOS Induction By Ni²⁺

To analyze whether iNOS was involved in the NO production induced by Ni²⁺, we measured nitrite concentrations in the presence or absence of the NOS inhibitors, NG-nitro-L-arginine methyl ester (L-NAME, eNOS inhibitor) and L-N₆-(1-iminoethyl)lysine (L-NIL, iNOS inhibitor). As shown in Figures 4A and B, L-NIL, but not L-NAME, significantly inhibited NO production by MDF stimulated with Ni²⁺ (Fig. 4A) or Ni²⁺ and IL-1 β (Fig. 4B). We also measured iNOS mRNA expression levels by quantitative RT-PCR (Fig. 4C). IL-1 β alone induced traceable amounts of iNOS mRNA expression. Ni²⁺ induced significantly higher expression levels of iNOS mRNA, and costimulation with Ni²⁺ and IL-1 β greatly induced the expression of iNOS mRNA. Moreover, iNOS protein was detected in MDF stimulated with Ni²⁺, and a higher amount of iNOS protein was detected by costimulation with Ni²⁺ and

IL-1 β (Fig. 4D). No iNOS protein was detected in MDF with medium and IL-1 β .

These results indicated that iNOS was induced by Ni²⁺ in MDF.

HIF-2 α , But Not HIF-1 α , Mediates iNOS Induction and NO Production

Because HIF mediates various cellular events induced by Ni (Ke and Costa, 2006), and iNOS expression is regulated by HIF (Melillo et al., 1995), we analyzed the effects of HIF- α inhibitors. As shown in Figures 5A and B, nitrite concentrations were significantly decreased in the presence of the HIF-2 α inhibitor, but not the HIF-1 α inhibitor, regardless of stimulation with Ni²⁺ alone (Fig. 5A) or with Ni²⁺ and IL-1 β (Fig. 5B). Moreover, the HIF-2 α inhibitor significantly decreased the expression levels of iNOS mRNA induced by Ni²⁺ alone or Ni²⁺ and IL-1 β (Fig. 5C). iNOS protein was also decreased in the presence of HIF-2 α inhibitor (Fig. 5D). No additive effects of HIF-1 α and HIF-2 α inhibitors were detected (data not shown). The expression of iNOS mRNA was dependent on HIF-2 α even with shorter period (8 h, data not shown).

These results indicated that HIF-2 α , but not HIF-1 α , was involved with iNOS induction and NO production by Ni²⁺-stimulated MDF.

NO Production Induced By Co²⁺

We analyzed other metal cations that have been reported as metal allergens. We tested Co²⁺, Cr²⁺, and Cu²⁺. Palladium (Pd) has also been reported as a metal allergen, and we previously showed that Ni and Pd exhibited cross reactivity (Kinbara et al., 2011a). However, because of its low solubility in neutral pH, we could not test Pd²⁺. No nitrite was detected in the culture supernatant of MDF stimulated with Cr²⁺ or Cu²⁺ (data not shown). As shown in Figure 6A, Co²⁺ had cytotoxic effects on MDF. In the presence of 100 μM of Co²⁺, MDF proliferation after 4 days of culture was approximately 32% of the medium (0 μM of Co²⁺). Even at a lower concentration (12.5 μM), cell proliferation was approximately 60%. Nitrite concentrations increased in a Co²⁺ dose-dependent manner up to 50 μM and significantly decreased with 100 μM of Co²⁺ (Fig. 6B). When cells were costimulated with Co²⁺ and IL-1 β , NO production was significantly augmented. Nitrite concentrations with 100 μM of Co²⁺ and IL-1 β were as same as those with 50 μM of Co²⁺ and IL-1 β . The inhibitor of HIF-2 α , but not HIF-1 α , significantly decreased NO production and the expression of iNOS mRNA induced by Co²⁺ alone or Co²⁺ and IL-1 β (Figs. 6C and D).

These results indicated that Co²⁺ also induced iNOS induction and NO production by MDF via the HIF-2 α -dependent pathway.

iNOS Inhibitor Augments, But HIF-2 α Inhibitor Attenuates Allergic Inflammation in Ni Allergy Mouse Model

Finally, we analyzed the *in vivo* effects of iNOS and HIF- α inhibitors on Ni allergy mouse model. Each inhibitor was

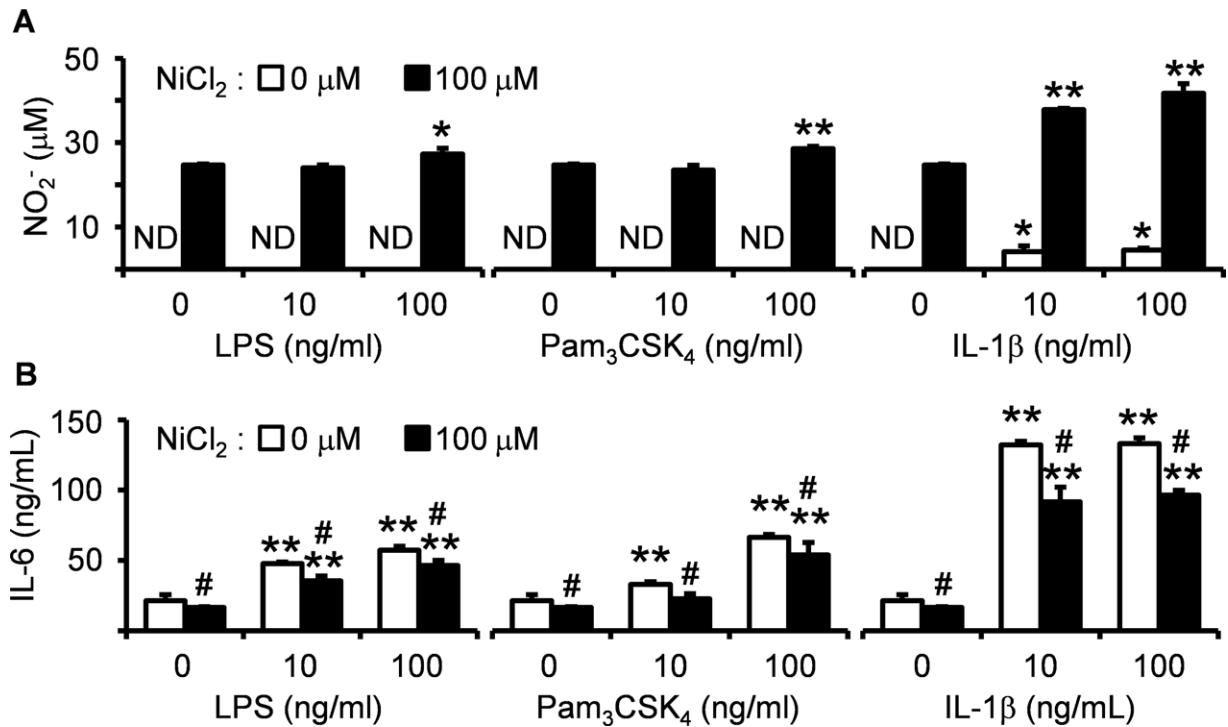


FIG. 3. IL-1 β augments NO production induced by Ni²⁺. Cells were cultured in medium containing 10% FBS with the indicated amount of NiCl₂ at a final volume of 500 μ l for 4 (A) or 1 (B) day. (A) Nitrite concentrations in the culture supernatants were measured with Griess reagent. (B) IL-6 concentrations in the culture supernatants were measured by ELISA. The results were expressed as the mean $\pm$ SD of a single experiment performed in triplicate. ND, not detected. * p < 0.05, ** p < 0.01, significantly different from no stimulation (0 ng/ml). # p < 0.05, significantly different from 0 μ M of NiCl₂.

coadministered with Ni at elicitation step. As shown in Figure 7, ear swelling was significantly increased in the presence of 100 μ M of iNOS inhibitor, L-NIL. Surprisingly, HIF-2 α inhibitor, but not HIF-1 α inhibitor, significantly inhibited the ear swelling.

These results indicated that iNOS and HIF-2 α showed anti- and proinflammatory effects on the Ni allergy mouse model, respectively.

DISCUSSION

We showed that iNOS expression and NO production were induced by Ni²⁺ in MDF via the HIF-2 α -dependent pathway. Several studies have reported Ni-induced NO production *in vivo* and *in vitro* (Cruz *et al.*, 2004; Gupta *et al.*, 2000). Ni is a well-known hypoxia-mimicking agent that activates HIF pathways (Ke and Costa, 2006). iNOS is a typical HIF target gene (Melillo *et al.*, 1995). Ni oxide nanoparticles, as well as Ni²⁺, induced HIF-1 α accumulation and its nuclear translocation in the human lung epithelial cell line NCI-H460 (Pietruska *et al.*, 2011). Primary cells, such as human endothelial cells (HUVEC) and human lung fibroblasts, also showed the activation of HIF pathways by stimulation with Ni²⁺ (Brant and Fabisiak, 2009; Viemann *et al.*, 2007). These findings support our present results that Ni²⁺ induced iNOS expression in MDF via a HIF-dependent mechanism. To the best of our

knowledge, this is the first study to show the Ni-HIF-iNOS axis directly.

Although HIF-1 α and HIF-2 α have overlapping functions, many studies have reported unique and sometimes opposing roles for both HIF- α subunits. Takeda *et al.* (2010) reported the opposing roles of HIF-1 α and 2 α in NO production by mouse peritoneal macrophages under hypoxia conditions in which HIF-1 α induced iNOS expression, but HIF-2 α regulated arginase 1 expression, which catabolizes L-arginine and competes with iNOS for their common substrate. Although the cell type was different, their report conflicts with our present results that HIF-2 α , but not HIF-1 α , regulated iNOS expression in MDF. Several studies have reported differential stabilities between HIF-1 α and 2 α . HIF-1 α is transiently stabilized under low hypoxia (1% O₂), whereas HIF-2 α is stabilized under moderate hypoxia (5% O₂) during prolong periods (Holmquist-Mengelbier *et al.*, 2006). In this study, iNOS expression induced by Ni²⁺ was dependent on HIF-2 α in not only longer (3 days) but also shorter (8 h) periods of time. The difference between “actual hypoxia” and “a hypoxia-mimicking agent” may affect the preference of HIF- α . Moreover, the different regulatory mechanisms of HIF- α have also been reported. The efficiency of HIF- α hydroxylation by PHDs and HIF asparaginyl hydroxylase was shown to differ between HIF-1 α and HIF-2 α (Appelhoff *et al.*, 2004; Koivunen *et al.*, 2004). Koh *et al.* (2011) reported that hypoxia-associated factor

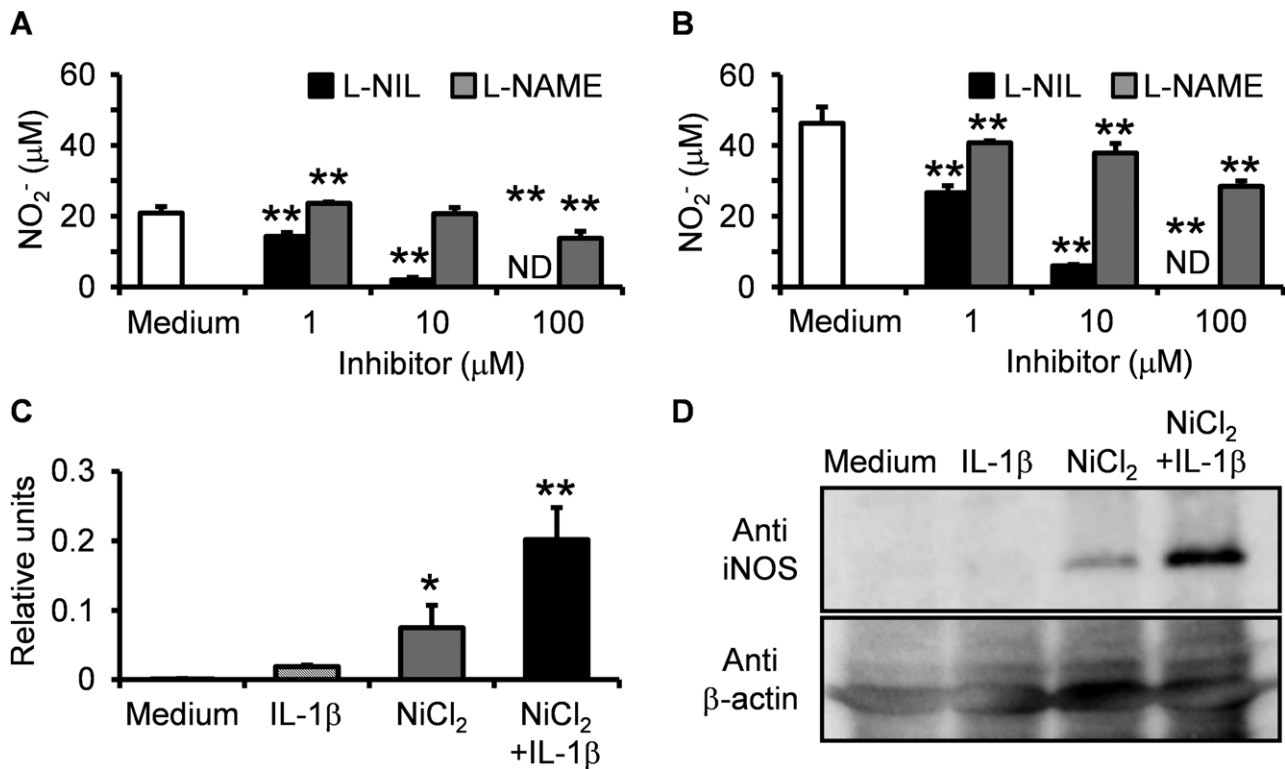


FIG. 4. iNOS is involved with NO production stimulated with Ni²⁺ and IL-1β. Cells were cultured in medium containing 10% FBS with the indicated amount of stimuli. (A and B) Cells were stimulated with 100μM NiCl₂ and the NOS inhibitors, L-NIL or L-NAME, in the presence (B) or absence (A) of 10ng/ml of IL-1β for 4 days. Nitrite concentrations were measured with Griess reagent. (C) Cells were stimulated with 100μM of NiCl₂, 10ng/ml of IL-1β, or both for 3 days. iNOS mRNA expression levels were determined by quantitative RT-PCR. The results were expressed as relative units after normalization by the GAPDH level. The results were expressed as the mean ± SD of a single experiment performed in triplicate. **p* < 0.05, ***p* < 0.01, significantly different from the medium. (D) Cells were stimulated with 100μM of NiCl₂, 10ng/ml of IL-1β, or both in a final volume of 10ml for 4 days. The whole cell lysate (50 μg protein/well) was applied to Western blotting.

mediated HIF-1α degradation but not HIF-2α activation. It is important to further investigate the stabilization and activation mechanisms of HIF-α induced by Ni.

IL-1β significantly augmented NO production by MDF stimulated with Ni²⁺. On the other hand, only slight augmentations were induced by the TLR ligands, LPS and Pam₃CSK₄. IL-6 production was also higher in MDF stimulated with IL-1β than with the TLR ligands. IL-1R and TLR share many signal transduction molecules, such as NF-κB and mitogen-activated protein kinase (MAPK) (O'Neill and Bowie, 2007), which suggests that signal strength, but not signal type, contributes to this augmentation. We previously reported that LPS reduces the minimum concentration of Ni to induce allergic inflammation in the elicitation step (Kinbara *et al.*, 2011b). LPS is a well-known inducer of IL-1β (Alheim and Bartfai, 1998), indicating that IL-1β induced by LPS cooperates with Ni in the elicitation step. The interaction between the HIF, MAPK, and NF-κB pathways has been reported, suggesting that the HIF signal induced by Ni and MAPK and/or the NF-κB signal induced by IL-1β have synergistic/additive effects.

iNOS inhibitor, L-NIL, augmented significantly ear swelling in Ni allergy mouse model, indicating that NO has

anti-inflammatory effects in our mouse model. This result agrees with other studies, which reported anti-inflammatory effects of NO. Sugita *et al.* (2010) reported that CHS response to hapten increased by the application of L-NIL, and iNOS expressed in cutaneous DC suppressed the migration and survival of DCs. NO-releasing hydrocortisone shows faster and greater protective effects than its parental compound (Hyun *et al.*, 2004). The cell adhesion molecules were downregulated by NO donor in HUVEC and smooth muscle cells (Shin *et al.*, 1996). Although NO is highly diffusible, the biological effects of NO are not restricted to the site of its production. NO metabolites, such as the low molecular weighted S-nitrosothiols, S-nitrosylated proteins, and nitrosyl-metal complexes, can act as long-distance NO vehicles (Bogdan, 2001). These observations suggest that NO derived from Ni-stimulated dermal fibroblasts shows anti-inflammatory effects in Ni allergy.

On the other hand, HIF-2α inhibitor reduced significantly ear swelling in Ni allergy mouse model. This surprising result is not keeping with the results that Ni-stimulated MDF produces NO via HIF-2α-dependent manner, and the inhibitor for iNOS augments ear swelling in Ni allergy mouse model. It was reported that mice lacking HIF-2α in myeloid cells are

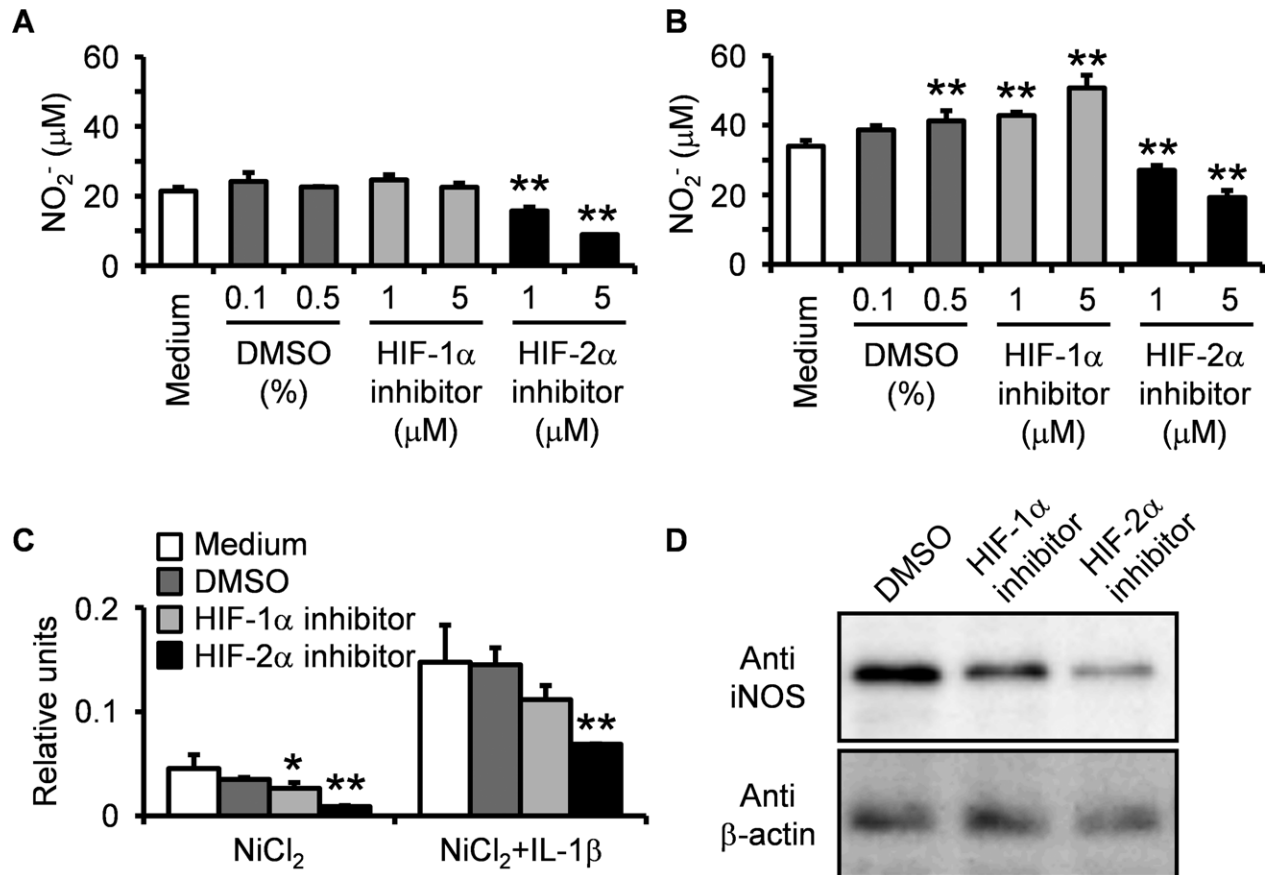


FIG. 5. HIF-2 α is involved with NO production stimulated with Ni²⁺ and IL-1 β . Cells were cultured in medium containing 10% FBS with the indicated amount of stimuli. (A and B) Cells were stimulated with 100 μ M NiCl₂ and the indicated amount of HIF-1 α or 2 α inhibitors in the presence (B) or absence (A) of 10 ng/ml of IL-1 β for 4 days. Dimethyl sulfoxide (DMSO) was used as the vehicle control. Nitrite concentrations were measured with Griess reagent. (C) Cells were stimulated with 100 μ M NiCl₂ and 5 μ M of HIF-1 α or 2 α inhibitors in the presence or absence of 10 ng/ml of IL-1 β for 3 days. A total of 0.5% of DMSO was used as the vehicle control. iNOS mRNA expression levels were determined by quantitative RT-PCR. The results were expressed as relative units after normalization by the GAPDH level. The results were expressed as the mean $\pm$ SD of a single experiment performed in triplicate. * p < 0.05, ** p < 0.01, significantly different from the medium. (D) Cells were stimulated with 100 μ M of NiCl₂ and 10 ng/ml of IL-1 β with 5 μ M of HIF-1 α or 2 α inhibitors in a final volume of 10 ml for 4 days. DMSO was used as the vehicle control. The whole cell lysate (50 μ g protein/well) was applied to Western blotting.

resistant to endotoxin shock and display a significantly lower inflammatory responses to cutaneous and peritoneal irritants (Imtiyaz *et al.*, 2010). Bone marrow-derived macrophages from the conditional knockout mice produce significantly lower amount of proinflammatory cytokines, such as IL-1 β , IL-6, and IL-12, indicating that HIF-2 α regulates proinflammatory cytokines expression. These observations suggest that, even though the HIF-2 α inhibitor downregulates the NO production by Ni-stimulated MDF, effects of lower amount of NO are masked by effects of lower amount of proinflammatory cytokines. Further studies will be needed to elucidate the precious roles of HIF-2 α in the pathogenesis of Ni allergy.

IL-6 production decreased significantly in the presence of Ni even though costimulation with TLR ligands or IL-1 β . Although we did not rule out the precious mechanisms, it may have been caused by the cytotoxic effects of Ni. On the other hand, Gao *et al.* (2010) reported that Ni induces IL-6

production by human lung fibroblasts. They also showed that IL-6 production increased significantly by costimulation with Ni and TLR2 ligand. This discrepancy is possibly caused by experimental system. They used human lung fibroblasts with serum-free medium. It is well known that serum affects cytokine production. Further studies are needed to verify the effects of Ni on the IL-6 production.

Dermal fibroblasts regulate DCs function by producing inflammatory cytokines and prostanoids (Saalbach *et al.*, 2010; Schirmer *et al.*, 2010), indicating that dermal fibroblasts are one of the important players in the pathogenesis of skin inflammation. Because Ni is challenged intradermally to ear pinnae, it can directly stimulate the dermal fibroblasts in our mouse model (Sato *et al.*, 2007). *In vitro* study with human full-thickness skin shows that a small but considerable amount of Ni is able to penetrate the stratum corneum and reach at dermis (Fullerton *et al.*, 1988). Moreover, volunteers cutaneously

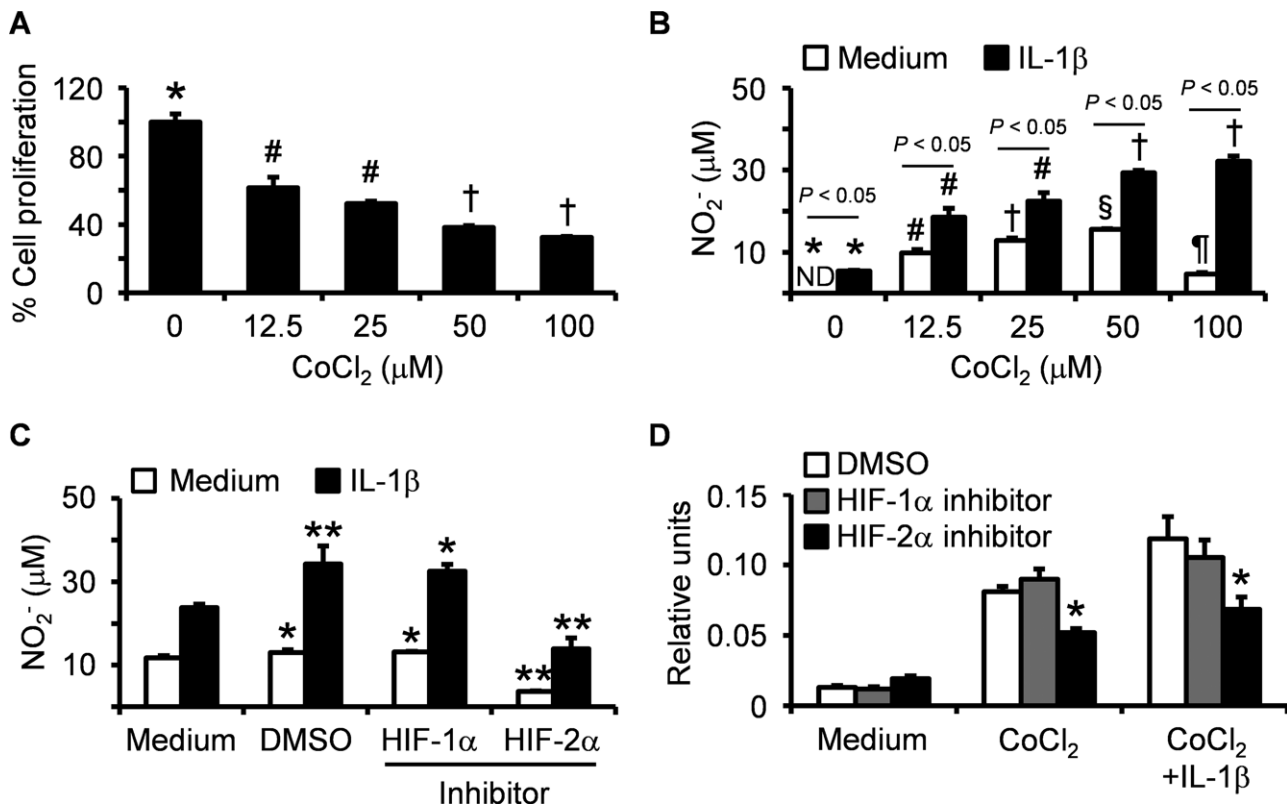


FIG. 6. NO production by MDF stimulated with Co²⁺. Cells were cultured in medium containing 10% FBS with the indicated amount of stimuli. (A) Cells were stimulated with the indicated amount of CoCl₂ for 4 days. Cell proliferation was measured by a MTT assay. The results were expressed as % cell proliferation after being normalized to the values with 0 μM CoCl₂. Means without a common symbol differ, $p < 0.05$. (B) Cells were stimulated with the indicated amount of CoCl₂ in the presence or absence of 10 ng/ml of IL-1β for 4 days. Nitrite concentrations were measured with Griess reagent. The results were expressed as the mean $\pm$ SD of a single experiment performed in triplicate. ND, not detected. Means without a common symbol differ between each group. (C) Cells were stimulated with 50 μM CoCl₂ and 5 μM of HIF-1α or 2α inhibitors in the presence or absence of 10 ng/ml of IL-1β for 4 days. A total of 0.5% of DMSO was used as the vehicle control. Nitrite concentrations were measured with Griess reagent. The results were expressed as the mean $\pm$ SD of a single experiment performed in triplicate. * $p < 0.05$, ** $p < 0.01$ significantly different from the medium. (D) Cells were stimulated with 50 μM CoCl₂ and 5 μM of HIF-1α or 2α inhibitors in the presence or absence of 10 ng/ml of IL-1β for 3 days. A total of 0.5% of DMSO was used as vehicle control. iNOS mRNA expression levels were determined by quantitative RT-PCR. The results were expressed as relative units after normalization by the GAPDH level. The results were expressed as the mean $\pm$ SD of a single experiment performed in triplicate. * $p < 0.05$, significantly different from DMSO.

exposed to Co were shown to have higher urinary Co concentrations (Scansetti *et al.*, 1994). These observations indicate that Ni and Co can directly stimulate dermal tissues and support the involvement of dermal fibroblasts in the pathogenesis of metal allergies. On the other hand, as a front line of biological defense, epidermal cells, such as Langerhans cells and keratinocytes, are likely to be the first responder to Ni (Ross and Reske-Kunz, 2001). These cells express iNOS mRNA at elicitation step of CHS induced by contact allergen 2,4-dinitrofluorobenzene (Ross *et al.*, 1998). Ni also induces the expression of iNOS in skin-derived DC (Cruz *et al.*, 2004). Although Ni induces inflammatory cytokines and cell adhesion molecules in human and mouse keratinocytes (Corsini *et al.*, 1998; Guéniche *et al.*, 1994), it has not been clarified whether Ni induces iNOS expression and NO production in the keratinocytes. Further investigations are needed to elucidate the interaction between Ni and skin tissues including epidermis and dermis.

Because we have the Ni allergy mouse model and it is relatively easy to progress to *in vivo* experiments, we used MDF in this study. On the other hand, it is important to evaluate whether human dermal fibroblasts respond to Ni same as MDF. The relevance of our data obtained in MDF for the pathogenesis of human Ni allergy needs to be validated.

The only effective method to prevent metal allergies is to reduce or avoid metal exposure (Thyssen and Menné, 2010). However, it is not easy to identify and avoid all metal allergens in various daily items. In this study, we clearly showed that two major metal allergens, Ni and Co, induced NO production by dermal fibroblasts. Although the mechanisms are still unclear, the modulation of this multifunctional gas mediator may help prevent a metal allergy. Further investigations are needed to elucidate the crucial roles of NO derived from metal-stimulated dermal fibroblasts in the pathogenesis of metal allergies.

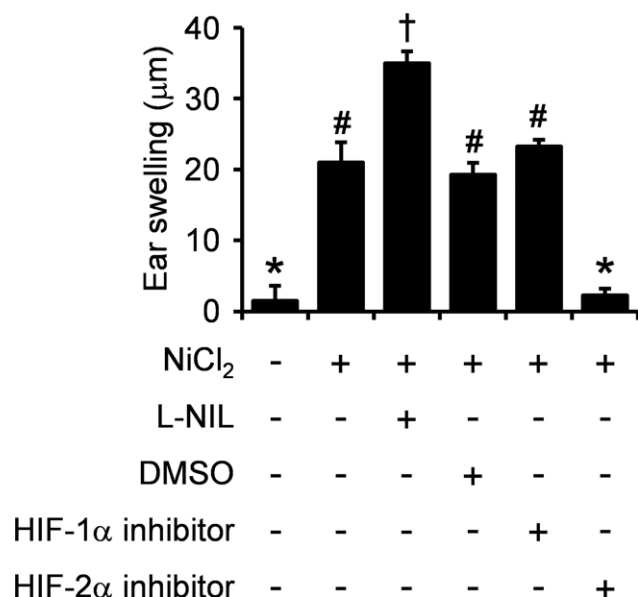


FIG. 7. Effects of iNOS and HIF- α inhibitors on the allergic inflammation in Ni allergy mouse model. Mice were sensitized with an ip injection of 250 μ l of solution containing 0.5mM NiCl₂ and 0.5 μ g/ml LPS. Ten days after sensitization, ear pinnae were challenged with an intradermal injection of 20 μ l of 1mM NiCl₂ with 100 μ M of L-NIL and 5 μ M of HIF-1 α or 2 α inhibitor. A total of 0.5% of DMSO was used as vehicle control to HIF- α inhibitors. Ear swelling was measured at 48 h after challenge. The results were expressed as the mean $\pm$ SD of four pinnae. Means without a common symbol differ, $p < 0.01$.

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