



Dual effect of nickel on L-arginine/nitric oxide system in RAW 264.7 macrophages

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ABSTRACT

The immunogenic mechanisms of the potent contact allergen nickel are not completely clear. Nitric oxide (NO) serves as a fundamental signalling and effector molecule in the immune system, but little is known about its possible role in immune reactions elicited by nickel. We investigated the effects of nickel on the L-arginine/inducible NO synthase (iNOS) system in a murine macrophage cell line, RAW 264.7. Both LPS-stimulated and non-stimulated RAW 264.7 cells were incubated in the presence of 0–100 μ M nickel sulphate for 24 h. Subsequently, NO production, iNOS protein expression, L-arginine uptake and gene expression of iNOS and cationic amino acid transporter systems (CAT) were measured. We found that 100 μ M NiSO₄ increased LPS-induced nitrite production as well as the formation of [³H]-L-citrulline from [³H]-L-arginine in the RAW 264.7 cells. Correspondingly, the expression of iNOS gene and protein was also remarkably enhanced. Nevertheless, nickel had an inhibitory effect on L-arginine transport which disappeared gradually upon LPS-stimulation in parallel with an increase in NO output. LPS was found to significantly amplify CAT-3 as well as CAT-2 mRNA expression, mirroring the increase in L-arginine transport. In the range of 1–10 μ M, NiSO₄ did not have any additional effect on CAT mRNA expression, but at 100 μ M it was able to enhance CAT-1 and CAT-3 mRNA expression upon LPS stimulation.

Our data indicate that nickel interferes with macrophages' L-arginine/NOS system on multiple levels. Considering the potent biological effects of NO, these influences may contribute to nickel toxicity.

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1. Introduction

Nickel represents the most common contact allergen in humans [1], but the factors underlying its strong immunogenicity are not completely clear. Nickel is capable of eliciting both immediate and delayed type hypersensitivity reactions in skin and a potential for systemic allergic reactions is also described. Besides being an allergen, nickel has been shown to exhibit immunosuppressant effects [1].

Nickel-reactivity of immune cells involves several mechanisms. The recognition pathway in specific T-cell response incorporates the trimolecular complex of MHC II molecules, hapten-modified peptides and T-cell receptors [2]. Besides acting as a conventional recall antigen, nickel may directly link T-cell receptors and the MHC in a peptide-independent manner, analogous to superantigens [3]. The potential mechanisms of nickel on the immune system are discussed in detail by Hostynek [1]. Nickel has also been shown to activate T cells independently from MHC I or II [4,5], suggesting that alternative mechanisms

may be involved. For example, a recent study has demonstrated that nickel directly activates human TLR4, a receptor for lipopolysaccharide (LPS) [6].

Nitric oxide (NO) serves as a fundamental signalling and effector molecule in the immune system [7]. NO is synthesised from L-arginine by a family of enzymes known as the nitric oxide synthases (NOS). In the immune system, the most important isoform is inducible NOS (iNOS). A wide variety of immune stimulants like LPS, IFN γ , IL-1, and TNF are potent inducers of iNOS. The activity of iNOS is primarily regulated by the synthesis of enzyme proteins.

In addition, the rate of NO synthesis is influenced by the availability of L-arginine [7–10]. The cellular arginine supply depends on the activity of cationic amino acid transporter (CAT) systems in the cell membrane [11,12]. Three separate genes encode the CAT-1, CAT-2 and CAT-3 proteins, respectively, which are known to exhibit cationic L-amino acid transport activity [13]. While CAT-1 is almost ubiquitously expressed [13], CAT-2 or its splice variant CAT-2B mRNA up-regulation along with stimulated NO synthesis has been demonstrated in RAW 264.7 cells [14,15], murine bone marrow-derived macrophages [16] and rat alveolar macrophages [17].

Few studies have attempted to delineate a potential role of NO in immune reactions elicited by nickel [18–20]. For one, increased iNOS immunoreactivity has been described in nickel-positive skin patch test sites [19].

Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; CAT, cationic amino acid transporter.

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We aimed to investigate the effects of nickel on the induction of iNOS, NO synthesis and L-arginine uptake in a murine macrophage cell line RAW 264.7. Recent evidence suggests that TLR4 of antigen presenting cells is important in nickel induced reactions [6]. The RAW 264.7 macrophages were selected as model since iNOS, L-arginine metabolism and transport systems have been well characterised in these cells upon stimulation [14,15,21,22].

2. Materials and methods

2.1. Cell culture

All reagents were purchased from Sigma-Aldrich unless stated otherwise. The murine macrophage cell line RAW 264.7 (obtained from American Type Culture Collection, LGC Promochem, Sweden), was maintained in DME medium supplemented with 10% FCS at 37 °C with 10% CO₂. For experiments, the cells were suspended at a density of 0.2×10^6 /ml in 24-well plates in 6 parallels. The following day, the cells were stimulated with 1 µg/ml LPS from *Salmonella typhimurium* (Alexis Biochemicals, Switzerland) and treated with 1, 10, 100 or 1000 µM nickel sulphate for 24 h. Samples without LPS and nickel served as controls.

2.2. Cell viability

The RAW 264.7 cells were incubated in the presence of the study compounds for 24 h. Viability of the cells was determined in two ways: (i) by the trypan blue dye exclusion test under a light microscope and (ii) by a commercial kit (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay, Promega) measuring formazan generation from tetrazolium compound MTS according to the manufacturer's instructions. Briefly, for the last 2 h of the 24-h incubation period in the 96-well cell culture plates, the kit reagents were added to the cells. The absorbance of the formazan was measured colourimetrically at 490 nm.

2.3. Nitrate and nitrite accumulation (Griess reaction)

The RAW 264.7 cells were incubated in the presence of the study compounds for 24 h. Thereafter, the culture supernatants were discarded and replaced with 250 µl of fresh medium containing the same compounds. After 4 h the medium was harvested and deproteinised with 30% (wt/vol) ZnSO₄, centrifuged at 4000 rpm for 5 min and 200 µl of the resulting supernatants were then transferred to the tubes containing 0.5 g of Cd beads. Samples were incubated overnight with agitation at room temperature, transferred to clean tubes and centrifuged again. Thereafter, 25 µl of water was mixed with 75 µl of the sample and 100 µl of Griess reagent was added. Absorbance at 540 nm was measured in a microplate reader. The nitrite concentration was determined with reference to a sodium nitrite standard curve.

2.4. L-Citrulline formation

The RAW 264.7 cells were incubated in the presence of the study compounds for 24 h. Thereafter, the culture supernatants were discarded and replaced with 100 µl Hanks' balanced salt solution (HBSS) containing the same compound and 100 nM of L-[2,3,4,5-³H] arginine (58 Ci/mmol; Amersham Biosciences, UK), and were then incubated for 4 hours. The cells were disrupted by three cycles of quick freezing and thawing. 40 µl of the suspension was mixed with 167 µl of methanol before being centrifuged at 22,000 rpm for 15 min. L-Citrulline was quantified by using the HPLC system with Allure Acidix column (250 × 4.6 mm i.d., Restek Corporation) and the radiochemical analyser Packard 150 TR [23].

2.5. Detection of iNOS by Western blot

The RAW 264.7 cells (0.2×10^6 /ml) were cultured with 10 ml of medium in 100 mm tissue culture dishes. Stimulated cells were harvested after the 24 h induction period with or without LPS and various concentrations of nickel sulphate. Fifteen micrograms of cytosolic protein from each sample was separated by 8% SDS-PAGE (Biometra GmbH) and electro-transferred to a PVDF membrane. Anti-iNOS rabbit polyclonal antibody (1:2000; Santa Cruz Biotechnology Inc, USA) was used as a primary antibody. Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:2500, DAKO Cytomation) was used as a secondary antibody. The blots were detected using an enhanced chemiluminescence kit (Amersham-Pharmacia Biotech, UK) and the luminogram of iNOS protein was obtained by exposure to Kodak film. The image was captured with a densitometer (BioRad) and analysed with Quantity One software. Crude cytosol from LPS (1 µg/ml) stimulated RAW 264.7 cells served as the positive control.

2.6. L-Arginine uptake

L-Arginine uptake was measured as described previously [24] with minor modifications. The RAW 264.7 cells were incubated in the presence of the study compounds for 24 h. Thereafter, the culture supernatants were replaced with 100 µl of HBSS containing the study compounds and 100 nM of [³H]-L-arginine, before being incubated for 15 min. HBSS was discarded and adherent cells were washed twice with PBS and resuspended in 100 µl of distilled water. The cells were disrupted by three cycles of freezing-thawing and the radioactivity was measured by liquid scintillation counting.

2.7. Protein content

The protein concentration of cell lysates was measured according to the Lowry method [25] using bovine serum albumin as a standard.

2.8. Gene expression

Total RNA was isolated from RAW 264.7 cells using Trizol reagent (Invitrogen Life Technologies, UK). cDNA synthesis was performed using the reverse transcriptase enzyme SuperScript III (Invitrogen Life Technologies). TaqMan Gene expression Assays and TaqMan Universal PCR Mastermix (Applied Biosystems) were used for quantitative real-time PCR (ABI PRISM 7900 HT Sequence Detection System equipment, PE Applied Biosystems, USA) to measure the expression levels of the following genes: iNOS (assay no Mm00440502_m1), CAT-1 (Mm01219060_m1), CAT-2 (Mm00432032_m1) and CAT-3 (Mm00500264_g1). Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as an endogenous reference gene (housekeeper; Mm01545399_m1). The PCR reaction of each sample was run in quadruplicate (four technical replicates) and an average threshold cycle (Ct) was calculated from these. Gene expression levels were analysed by comparing the values of ΔCt ($\Delta Ct = Ct \text{ of target gene} - Ct \text{ of housekeeper}$) transformed to a linear scale ($2^{-\Delta Ct}$).

2.9. Statistics

Data are presented as mean ± SEM of 4–6 parallel experiments. Analysis of variance (ANOVA) with Newman-Keuls post hoc test was used when variances were distributed parametrically and with Mann-Whitney U test in case of non-parametric distributions.

Table 1

Effects of nickel on cell proliferation and viability. Initially, 0.2×10^6 cells/ml were seeded and incubated in the presence of the indicated compounds for 24 h. Cells were counted using the trypan blue dye exclusion test for the assessment of viability ($n=4$). In another set of experiments, the mixture of tetrazolium compound MTS and phenazine methosulphate was added to the culture for the last 2 h and formazan product generation was measured colourimetrically at 490 nm ($n=6$). Data presented are means $\pm$ SEM.

LPS $\mu\text{g/ml}$	NiSO ₄ μM	Viability (%)	Formazan generation (AU)
0	0	97.2	1.60 (± 0.08)
0	1	99.1	2.53 (± 0.10)*
0	10	98.6	2.91 (± 0.26)*
0	100	84.9*	1.88 (± 0.34)
0	1000	12.7*	0.38 (± 0.03)*
1	0	86.5*	1.76 (± 0.30)
1	1	84.9	2.30 (± 0.08)**
1	10	76.6	2.49 (± 0.12)**
1	100	71.0**	2.51 (± 0.06)**
1	1000	17.1**	0.25 (± 0.01)**

* $p < 0.05$ vs. LPS 0 $\mu\text{g/ml}$ and NiSO₄ 0 μM ; ** $p < 0.05$ vs. LPS 1 $\mu\text{g/ml}$ and NiSO₄ 0 μM .

3. Results

3.1. Cell viability

For further studies, nickel was used in the range of 1–100 μM , since the presence of 1 mM nickel seriously compromised cellular viability (Table 1).

3.2. Effect of nickel on nitrates/nitrites accumulation

No detectable production of nitrates/nitrites by non-stimulated cells was found and nickel did not change this result (data not shown). Incubation of cells with LPS for 24 h significantly increased the levels of nitrates/nitrites. Co-treatment of cells with LPS and 100 μM nickel during 24 h further potentiated the stimulatory effect of LPS (Fig. 1).

3.3. Effect of nickel on NOS activity

In line with the previous experiment, there was no detectable NOS activity in non-stimulated macrophages with or without nickel exposure (data not shown). LPS-induced NOS activity was further augmented by 10 μM and 100 μM nickel (Fig. 2).

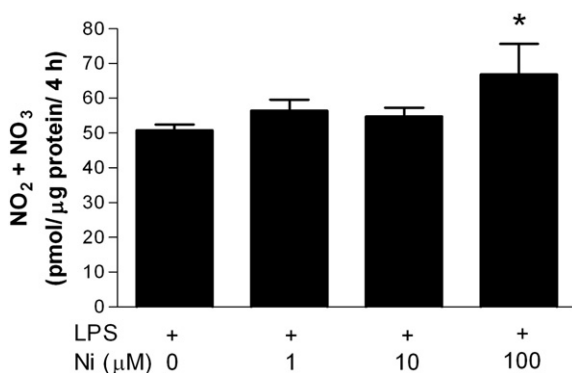


Fig. 1. Effect of nickel on nitrates/nitrites accumulation. RAW 264.7 cells were incubated with LPS 1 $\mu\text{g/ml}$ and 0–100 μM NiSO₄ for 24 h. Thereafter, the culture medium was changed and the accumulation of nitrites/nitrates after 4 h was measured. The results were normalised for protein content. The data are presented as mean $\pm$ SEM ($n=6$). * $p < 0.02$ vs. LPS only, Mann–Whitney U test.

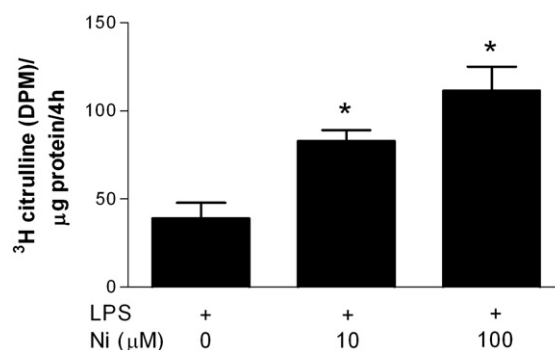


Fig. 2. Effect of nickel on [³H]-L-citrulline formation upon LPS stimulation. RAW 264.7 cells were incubated with LPS 1 $\mu\text{g/ml}$ and 0–100 μM NiSO₄ for 24 h. Thereafter, the culture medium was replaced with HBSS containing 100 nM [³H]-L-arginine for 4 h. Formed [³H]-L-citrulline was detected by HPLC. The results were normalised for protein content. The data are presented as means $\pm$ SEM ($n=6$). * $p < 0.0005$ vs. LPS only, Newman–Keuls test.

3.4. Effect of nickel on iNOS protein expression

Fig. 3 shows that iNOS expression was induced by LPS (line 2) whereas in the cytosolic extract of unstimulated cells iNOS-like immunoreactive material was not detectable (line 1). Co-treatment of cells with LPS and 100 μM nickel induced about 20-fold increase in iNOS expression when compared with LPS-stimulated cells (line 4).

3.5. Effect of nickel on L-arginine uptake

The uptake of L-arginine was linear over the time range of 5–15 min. Stimulation of cells with LPS significantly increased the uptake of L-arginine (Fig. 4). Nickel had an inhibitory effect on L-arginine uptake. In the case of non-stimulated cells, only the 100 μM concentration inhibited the uptake of L-arginine. In LPS-stimulated cells, nickel hampered L-arginine transport at concentrations of 1 and 10 μM but not at 100 μM .

3.6. Effect of nickel on the expression of iNOS and CAT mRNA

LPS greatly up-regulated iNOS mRNA expression in RAW 264.7 cells and nickel steadily augmented this, albeit reaching a statistically significant effect only at 100 μM (Fig. 5(A)). While LPS increased the mRNA expression of CAT-2 and CAT-3 and had the most profound effect on CAT-2, it only somewhat induced CAT-1 mRNA expression. The expression of distinct CAT mRNAs was dissimilarly influenced by nickel. Nickel in the range of 1–100 μM did not have any effect on LPS-induced CAT-2 mRNA expression, yet CAT-1 and CAT-3 mRNA expression was up-regulated by 100 μM nickel (Fig. 5(B–D)).

4. Discussion

In this work we investigated the effects of nickel on NO synthesis, iNOS protein expression and L-arginine transport in the RAW 264.7 cell line. Nickel severely compromised cell viability at concentrations higher than 100 μM . Therefore, higher concentrations of the compound were not included in the analysis. Toxic effects of nickel are well known, as several reports have documented that nickel can trigger cell apoptosis in the concentration range of 100–640 μM [26].

In our study, nickel affected the L-arginine/NO system in the concentration range of 1–100 μM . These concentrations are even lower than in previous reports, where nickel ranges from 0.1 to 1.5 mM have been used [27,28]. It is currently unknown what concentrations should be reached in the skin after epicutaneous nickel exposure to induce biological effects. In humans, the positive patch-test reactions to nickel sulphate occur at relatively high concentrations, i.e. 0.3 mM–

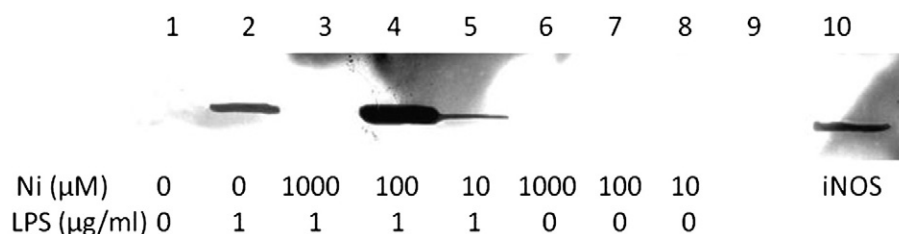


Fig. 3. Effect of LPS and nickel on iNOS expression in RAW 264.7 cells. The cells were incubated 24 h in the presence or absence of LPS 1 μg/ml and with NiSO₄ in different concentrations. Equal amounts of cytosolic proteins (15 μg) were applied on an 8% SDS-PAGE (lanes 1–8). Lane 9 signs the prestained molecular weight standards. Lane 10 – positive control. The presented results represent one of three independent experiments.

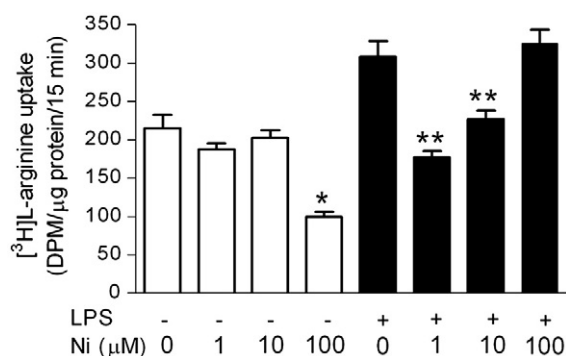


Fig. 4. Effect of nickel on L-arginine uptake into RAW 264.7 cells. The cells were incubated with or without LPS (1 μg/ml) and 0–100 μM NiSO₄ for 24 h. Thereafter, the culture medium was replaced with HBSS containing 100 nM [³H]-L-arginine and incubated for 15 min. The cells were washed twice, lysed and radioactivity taken up was detected by liquid scintillation counting. The results were normalised for protein content. The data are presented as means ± SEM (n=6). *p<0.005 vs. control, **p<0.005 vs. LPS only, Mann–Whitney U test.

150 M [29]. In a recent *in vivo* study in mice, 10 mM NiCl₂ was injected intracutaneously to induce as well as to challenge contact hypersensitivity [6]. Taken together, one can assume that the nickel concentrations used in the current study fall well into the clinically relevant range.

As NO is a gaseous and quickly metabolised molecule, it is difficult to directly measure its concentration. We used two indirect methods for measuring NO synthesis. Both of these demonstrated that nickel significantly potentiates the LPS-stimulated production of NO by RAW 264.7 macrophages. While the Griess assay showed that nickel is active at the concentration of 100 μM, the more sensitive radioactive citrulline method also demonstrated increased NOS activity by 10 μM nickel. These results were supported by the finding that 100 μM nickel significantly augmented the iNOS gene and protein expression.

Our data are in concordance with previous studies evaluating the effect of nickel in different macrophage types [20,30]. Thus, using the Griess assay, Tian and Lawrence showed that nickel enhanced NO production and iNOS protein expression in cytokine-stimulated murine macrophages in a dose-dependent manner [20]. It is important to stress that, in our study as well as in a previous report [30], nickel alone did

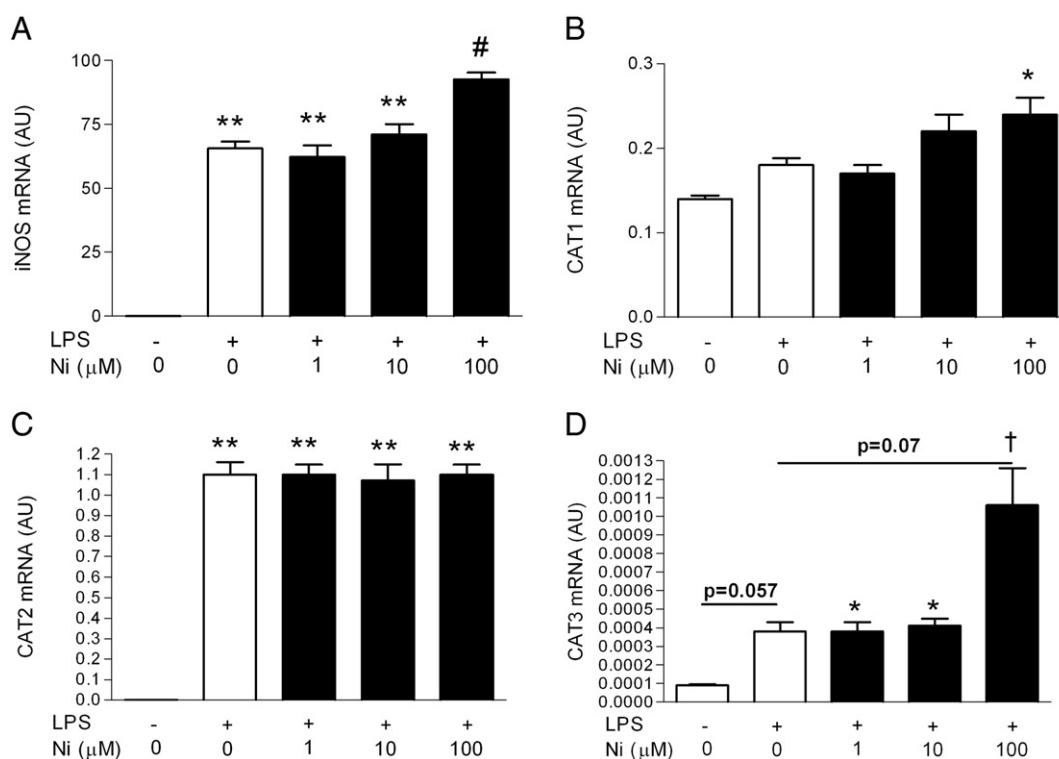


Fig. 5. Effect of nickel on the expression of iNOS and CAT mRNA. After the RAW 264.7 cells were incubated with or without LPS (1 μg/ml) and 0–100 μM NiSO₄ for 24 h, total RNA was isolated and cDNA synthesised. Using RT-PCR, the expression levels of iNOS, CAT-1, CAT-2 and CAT-3 mRNA were measured. The average threshold cycle (Ct) of the RT-PCR was calculated from four technical replicates. The values of ΔCt (ΔCt = Ct of target gene – Ct of housekeeper) transformed to a linear scale (2^{-ΔCt}) were compared. The data are presented as means ± SEM (n=4). *p<0.05 vs. no stimulation, **p<0.0005 vs. no stimulation, #p<0.0005 vs. LPS only, †p<0.02 vs. 1 and 10 μM nickel. (A), (B), (C) – Newman–Keuls test, (D) – Mann–Whitney U test.

not induce NO production in RAW 264.7 macrophages. Accordingly, at least in RAW 264.7 cells nickel acts as a co-inducer of iNOS. Still, in the foetal mouse skin dendritic cells, which expressed iNOS under basal conditions, nickel alone was sufficient to increase NO production [18].

A recent study has demonstrated that nickel directly activates human TLR4, which is known to mediate LPS-stimulated NO synthesis [6]. These results lend support to our findings by further implicating TLR4 in immune reactions to nickel. Despite the fact that nickel was not found to bind the murine TLR4 [6], our data clearly suggest that when RAW 264.7 cells are activated via TLR4 by LPS, nickel acts as a co-stimulator, at least in the arginine/NOS system. Likewise, LPS has been reported to promote cutaneous hypersensitivity reactions to nickel in mice, including nude (i.e. mature T cell deficient) mice [31–33]. Collectively, the current evidence unanimously demonstrates that nickel potentially enhances NO production by stimulated macrophages. Considering that NO is an important mediator in the immune system, we suggest that the ability of nickel to stimulate NO production contributes to its allergic properties. Further studies are needed to directly demonstrate whether this mechanism is operational in cases of allergic reactions to nickel.

Another significant finding in the current study was that nickel had a dual action on L-arginine transport. In most instances L-arginine transport seems to be coupled to NO synthesis, as several studies have demonstrated increased L-arginine transport in cases of stimulated NO production and transport proteins are usually up-regulated by the same stimulants, cytokines and cofactors inducing NOS [10,14,16,17,34,35]. Likewise, LPS enhanced the expression of CAT-2, as well as CAT-3 mRNA in our experiment, accompanied by stimulated L-arginine uptake. To our knowledge, the stimulatory effect of LPS on CAT-3 mRNA expression has not been described previously.

Unexpectedly, nickel had an inhibitory effect on L-arginine transport in RAW 264.7 cells under both basal and LPS-stimulated conditions. In LPS-stimulated cells the inhibitory effect of nickel appeared at lower doses. Since L-arginine transport was increased by 50% under LPS stimulation and also the expression of the three CAT mRNA was differently affected, it is difficult to draw firm conclusions on the variegated dose–response of nickel under basal and LPS-stimulated conditions. Interestingly, the inhibitory effect disappeared gradually with higher doses of nickel in parallel with an increase in NO output in LPS-stimulated cells. The reason for that seems to be further augmentation of L-arginine transporters by nickel, as 100 μ M nickel further amplified CAT-1 and CAT-3 gene expression and increased NO synthesis in combination with LPS stimulation.

Thus, nickel has at least two competitive functional effects on the L-arginine/NO system. The current evidence unanimously demonstrates that nickel potentially enhances NO production by stimulated macrophages. We propose that the inhibitory influence of nickel on L-arginine uptake in LPS-stimulated macrophages is counterbalanced by its ability to potentiate the CAT-1, CAT-3 and NO production.

The influence of nickel on L-arginine transport may have wider implications, as arginine is a substrate for two other enzymes – arginase and arginine decarboxylase. While the presence of functionally active arginine decarboxylase in mammalian cells remains obscure [36], arginase is an important regulator of cell division and NOS activity [7,22]. Thus, the inhibition of L-arginine transport by nickel may also influence arginase activity and the balance between arginase and NOS activities.

In conclusion, our data indicate that nickel interferes in the macrophage L-arginine/NOS system on multiple levels. Considering the important immunoregulatory effects of NO, these influences may contribute to the immune processes and toxicity elicited by nickel.

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The authors declare that they have no conflict of interest.

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