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Ni^{2+}, a Double-Acting Inhibitor of Neuronal Nitric Oxide Synthase Interfering with L-Arginine Binding and Ca^{2+}/Calmodulin-Dependent Enzyme Activation

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Ni^{2+}, a toxic and carcinogenic pollutant and one of the leading causes of contact dermatitis, is shown to inhibit neuronal nitric oxide synthase (nNOS) in a competitive, reversible manner with respect to the substrate L-arginine ($K_i = 30 \pm 4$ μM). The IC_{50} values were dependent on calmodulin (CaM) concentration, but proved independent of Ca^{2+}, tetrahydrobiopterin (BH_4) and other essential cofactors. Ni^{2+} also inhibited CaM-dependent cytochrome *c* reduction, NADPH oxidation, and H_2O_2 production by nNOS. Overall, the action profile of Ni^{2+} was suggestive of an unusual, double-acting inhibitor of nNOS affecting L-arginine-binding and Ca^{2+}/CaM-dependent enzyme activation.

Key Words: **neuronal nitric oxide synthase inhibition; calmodulin; nickel ions.**

Nickel, both in alloys and in the ionic (mainly Ni^{2+}) forms, is the focus of increasing biomedical and social attention because of its toxic and carcinogenic properties accounting for several pathological conditions affecting occupationally exposed populations. Concern is warranted by the significant mobility associated with natural nickel turnover and with human activity, which promotes rapid spread and accumulation of the element in the environment, including in food (1, 2).

Nickel is also a potent allergen, to a point that hypersensitivity to nickel-containing alloys represents the most common manifestation of allergic contact dermatitis (3).

Available evidence indicates that Ni^{2+} can damage genetic material (4–6), can interact with proteins, including enzymes (7, 8) and can promote and/or amplify lipid peroxidation (9).

Another somewhat overlooked property of Ni^{2+} emerges from scattered observations indicating that the metal can inhibit the activity of nitric oxide synthase (NOS) (10, 11), the enzyme deputed to the biosynthesis of the potent physiological modulator nitric oxide (NO). NOS occurs in at least three distinct isoforms termed neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) (12, 13). All isoforms contain a C-terminal reductase domain that binds NADPH, FAD and FMN, and an N-terminal oxygenase domain, where a heme group forms the catalytic center together with the binding sites for the substrate L-arginine and the cofactor tetrahydrobiopterin (BH_4). Electron flow from NADPH to the heme is triggered by the binding of Ca^{2+}-activated calmodulin (CaM), which induces proper alignment of the reductase and oxygenase domains and allows conversion of the substrate L-arginine into NO and L-citrulline. Besides NO synthesis, NOS can catalyze two additional reactions, namely reduction of molecular oxygen, yielding superoxide and H_2O_2, and reduction of cytochrome *c*. In the presence of Ca^{2+}/CaM the latter reaction is mediated by superoxide released from heme, whereas in the absence of Ca^{2+}/CaM heme is not reduced and cytochrome *c* reduction is brought about by a flavin-mediated process confined to the reductase domain.

Despite the possible pathological relevance of NOS inhibition, a detailed investigation of the effects of Ni^{2+} on NOS activity is apparently lacking. The present paper discloses a peculiar inhibition profile of nNOS by Ni^{2+}, highlighting the Ca^{2+}/CaM complex as a specific target of the metal.

MATERIALS AND METHODS

Materials

Recombinant rat nNOS and mouse iNOS were from Alexis Italia (Italy). Recombinant bovine eNOS was from Cayman (U.S.A.). Radiolabeled L-[U-^{14}C]arginine monohydrochloride (317 mCi/mmol)

Abbreviations used: NOS, nitric oxide synthase; nNOS, neuronal NOS; iNOS, inducible NOS; eNOS, endothelial NOS; CaM, calmodulin; BH_4, tetrahydrobiopterin.

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was obtained from Amersham Italia (Italy). NADPH, calmodulin (CaM), FAD, FMN, BH_4, $NiCl_2$, Ni SO_4, and all other reagents were obtained from Sigma (Italy).

NOS Assays

L-[^{14}C]Citrulline formation. NOS activity was determined by monitoring the conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline. For nNOS, aliquots (2 μl, 0.18 μg protein) of commercial recombinant enzyme were incubated for 15 min at 37°C in 0.1 ml of 20 mM Tris–HCl, pH 7 containing 0.5 mM NADPH, 250 μM calcium chloride, 10 μM BH_4, 10 μg/ml calmodulin, 5 μM FAD, 5 μM FMN and 1.57 μM L-[U-^{14}C]arginine. In all cases, each reaction was stopped by the addition of 0.9 ml of ice-cold 100 mM Hepes buffer containing 4 mM EDTA, pH 5.5, and was passed through 1 ml Dowex 50 (Na^+ form) column that retains L-arginine. The L-[^{14}C]citrulline generated was eluted with water and quantified by liquid scintillation counting.

For cofactor dependence inhibition studies, the concentrations used were 1.57 μM L-arginine, 100 μM $NiCl_2$, CaM from 1 to 40 μg/ml, FAD and FMN from 0.5 to 5 μM, BH_4 from 1 to 10 μM.

For isoform selectivity experiments, comparable NOS activities were used and the enzymes assayed under the same experimental conditions.

Reversibility of NOS inhibition was tested according to Garvey *et al.* (14). Briefly, nNOS was preincubated at 4°C for 10 min in the presence of cofactors with or without 100 μM inhibitor. The reaction was then started by the addition of L-arginine. Portions of the mixture were removed at fixed intervals and assayed for NOS activity.

H_2O_2 formation by nNOS. H_2O_2 formation by nNOS was determined by the ferrous thiocyanate assay (15). Briefly, commercial recombinant enzyme (1.35 μg) was incubated at 37°C for 10 min in 0.45 ml of a 50 mM triethanolamine/HCl buffer, pH 7, containing 0.5 mM NADPH, 3 μM $CaCl_2$ and 3 μg of CaM. Reactions were terminated by the addition of 225 μl of concentrated HCl. Solutions of 80 mM ferrous ammonium sulfate (30 μl) and 3 M potassium thiocyanate (45 μl) were added to final concentrations of 3.2 and 180 mM, respectively. Samples were incubated for 10 min at room temperature and the absorbance at 492 nm (A_{492}) was determined. Blank values were measured in the absence of the enzyme but with all cofactors present. Observed changes in A_{492} in the blank during readings did not exceed 15% of the initial value, and were duly considered in hydrogen peroxide determinations.

Cytochrome c reduction by nNOS. The initial rates of cytochrome *c* reductase activity of nNOS, in the absence and in the presence of $CaCl_2$ (2 mM), CaM (10 μg/ml) and $NiCl_2$ (500 μM), were determined spectrophotometrically at 30°C monitoring the absorbance at 550 nm as previously described (16).

NADPH oxidation by nNOS. The initial rates of NADPH oxidation by nNOS were measured spectrophotometrically at 37°C monitoring the absorbance at 340 nm as previously described (17). When required, L-arginine (1 mM) and $NiCl_2$ (500 μM) were added.

Data Analysis

All values were determined in triplicate and the means ± SEM from four separate experiments were calculated. Statistical analysis of data was performed by using the software of GraphPad Prism, version 2.00.

RESULTS

Incubation of nNOS with $NiCl_2$ resulted in a concentration-dependent inhibition of the enzyme activity (IC_{50} = 80 ± 4 μM), as determined by the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline (Fig. 1).

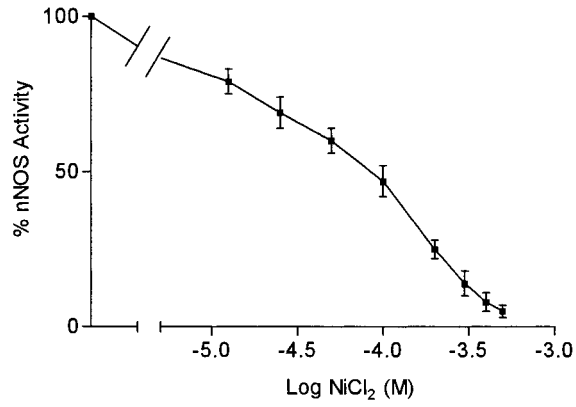

FIG. 1. Concentration-dependent inhibition of nNOS by $NiCl_2$. NOS activity was measured as the production of L-[^{14}C]citrulline from L-[^{14}C]arginine as described under Materials and Methods. Results are means ± SEM from four separate experiments.

Comparative assays on the various NOS isoforms indicated a more pronounced inhibition of nNOS and a lower activity toward iNOS (Fig. 2). No significant difference in the degree of inhibition of nNOS activity was found between $NiCl_2$ and $NiSO_4$ ruling out a nonspecific effect of the anion (Fig. 2). Accordingly $NiCl_2$, in the following indicated as Ni^{2+}, was preferably used throughout this study. That the inhibition was not due to a mere alteration of ionic strength was apparent from separate experiments showing that 15 mM NaCl did not affect the enzyme activity.

The progress curve for inhibited nNOS was linear, the onset of inhibition was rapid and L-citrulline formation increased almost linearly during a 15-min incubation time (data not shown), suggesting that inhibition by Ni^{2+} was reversible. Consistent with this conclusion, preincubation of nNOS with 100 μM Ni^{2+} for 10 min prior to initiation of the reaction with 1.57 μM L-arginine did not cause additional inhibition, ruling out any irreversible effect.

Initial-rate kinetic studies were carried out over a range of L-[^{14}C]arginine and Ni^{2+} concentrations to determine the nature of nNOS inhibition. Double-reciprocal plots of nNOS activity as a function of L-arginine concentration showed that Ni^{2+} inhibits L-citrulline formation in an essentially competitive manner (Fig. 3). Replots of data allowed determination of a K_i value of 30 ± 4 μM. In line with a basically competitive mechanism, inhibition of nNOS by 50 μM Ni^{2+} was reversed by increasing L-arginine concentration, complete suppression being observed at values greater than 50 μM. Notably, inhibition by 100 μM Ni^{2+} of citrulline formation from 1.57 μM L-[^{14}C]-arginine was not affected by 100 μM D-arginine.

Ni^{2+} was also found to inhibit H_2O_2 production by nNOS in the absence of L-arginine and tetrahydrobiopterin (BH_4) with an IC_{50} value of 45 ± 10 μM. Control

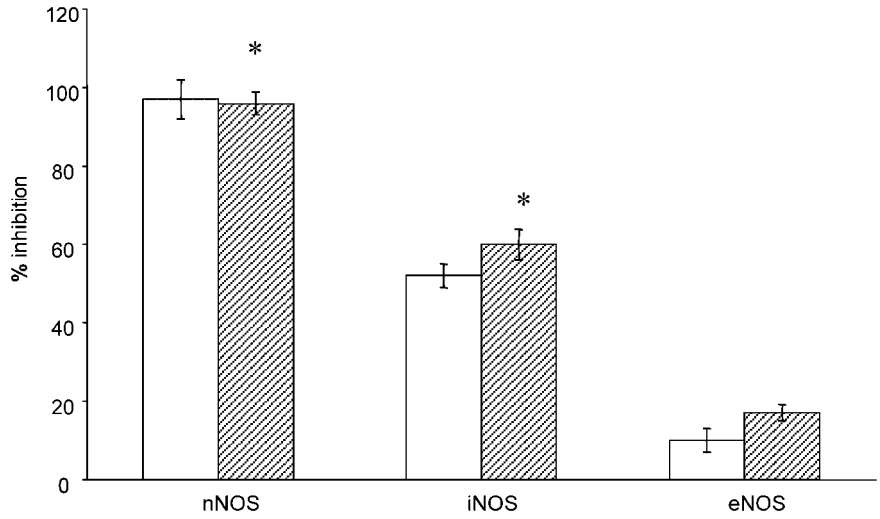

FIG. 2. Inhibition of NOS isoforms by $NiCl_2$ and $NiSO_4$. NOS activity of the different isoforms was measured as the production of L-[^{14}C]citrulline from L-[^{14}C]arginine as described under Materials and Methods. $NiCl_2$ (open bars), $NiSO_4$ (hatched bars). Results are means ± SEM from four separate experiments. Statistical significance: * $P < 0.01$, in other cases $P < 0.001$.

experiments indicated that Ni^{2+} does not affect H_2O_2 determination by the ferrous thiocyanate assay.

In subsequent experiments, the inhibitory effect of Ni^{2+} on L-citrulline formation from 1.57 μM L-[^{14}C]-arginine was investigated at various concentrations of FAD, FMN, Ca^{2+}, CaM and BH_4. The results showed a dependence of the inhibition on CaM concentration, IC_{50} values of 20 ± 3, 80 ± 4 and 130 ± 5 μM being determined with CaM concentrations of 1, 10, and 40 μg/ml, in that order.

The effect of Ni^{2+} on the nNOS-catalyzed reduction of cytochrome *c* by NADPH was then investigated in the absence or in the presence of Ca^{2+}/CaM. This reduction is much faster in the presence of Ca^{2+}/CaM which causes about 10-fold stimulation of the activity (6000 ± 100 versus 630 ± 64 nmol reduced cytochrome *c* min^{-1} mg $protein^{-1}$) (Fig. 4A). At a concentration of 500 μM, Ni^{2+} did not affect the CaM-independent cytochrome *c* reduction, whereas it inhibited the CaM-dependent activity by 57% (Fig. 4A).

Ni^{2+} caused also about 78% inhibition of NADPH oxidase activity of nNOS in the absence of L-arginine (Fig. 4B), whereas in the presence of L-arginine, under conditions in which NADPH oxidation rate is lowered by a factor of about 0.6 (18), it proved much less active (20%).

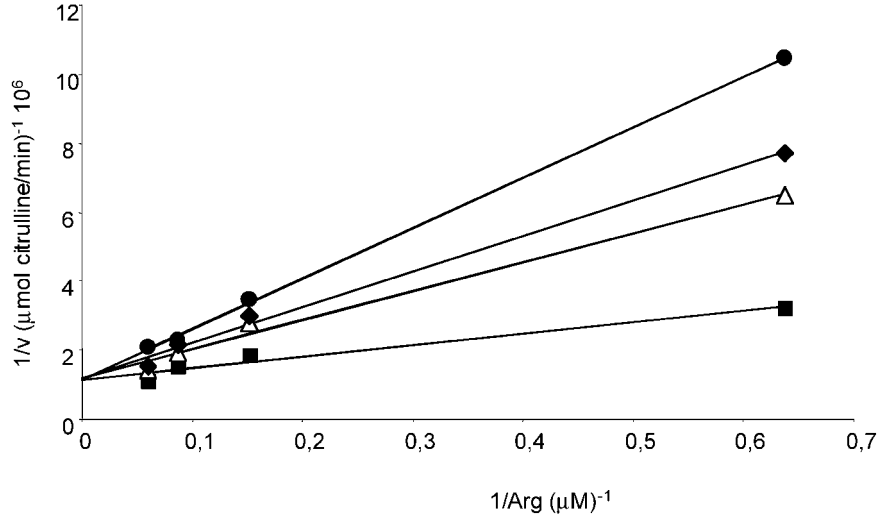

FIG. 3. Double reciprocal plot of nNOS activity as a function of L-arginine concentrations, in the presence of various concentrations of $NiCl_2$. Rates of L-[^{14}C]citrulline formation was measured as described under Materials and Methods in the absence (■) or in the presence of 25 (△) or 50 (◆) or 100 (●) μM $NiCl_2$. The plot shown is representative of three separate experiments.

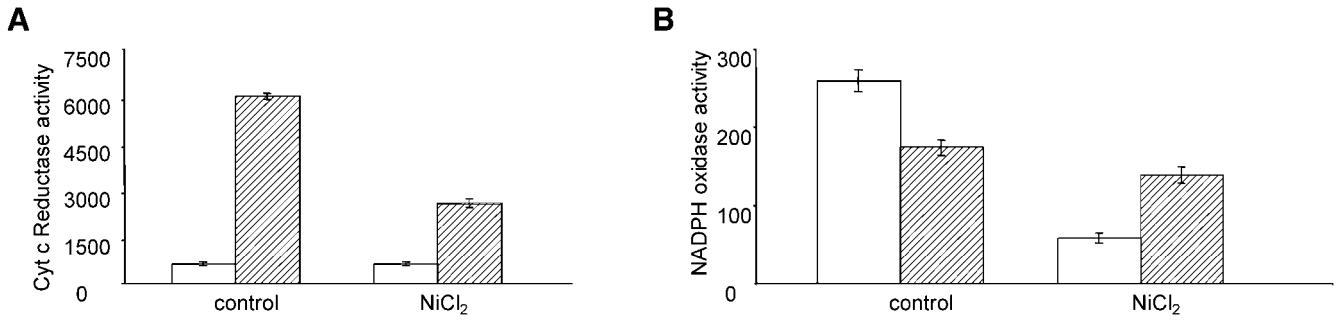

FIG. 4. Effect of $NiCl_2$ (500 μM) on cytochrome *c* reductase and NADPH oxidase activities of nNOS. (A) Cytochrome *c* reduction was assayed as described under Materials and Methods without Ca^{2+}/CaM (open bars) or in the presence of Ca^{2+}/CaM (hatched bars). Activities are expressed in nmol cytochrome *c* reduced min^{-1} mg $protein^{-1}$. (B) NADPH oxidation was assayed as described under Materials and Methods in the absence (open bars) and in the presence of 1 mM L-arginine (hatched bars). Activities are expressed in nmol NADPH consumed min^{-1} mg $protein^{-1}$. Results are means ± SEM from four separate experiments.

Spectrophotometric analysis showed that Ni^{2+} up to 1 mM causes barely appreciable changes in the absorption properties of recombinant nNOS, ruling out substantial effects on the low spin-high spin states of heme.

DISCUSSION

nNOS inhibition by Ni^{2+} displays features that differ from those of most other inhibitors investigated in detail so far, reflecting apparently an interfering effect on both L-arginine binding and Ca^{2+}/CaM-dependent enzyme activation. The competitive type of inhibition of L-citrulline production and the decreased inhibitory effect on NADPH oxidation in the presence of L-arginine are the main arguments supporting a direct interaction of Ni^{2+} with the L-arginine binding site of nNOS. A reasonable hypothesis is that the metal targets a negatively charged group within the L-arginine binding site and that L-arginine binding restores catalytic activity by simply displacing the bound Ni^{2+}. The lack of effect of D-arginine would rule out chelate formation between the amino acid and the metal as a possible underlying mechanism of inhibition.

The most noticeable feature of nNOS inhibition by Ni^{2+} is the marked dependence of IC_{50} values on CaM concentration. This observation, if considered in the light of the inhibitory effects of Ni^{2+} on Ca^{2+}/CaM-dependent cytochrome *c* reduction, NADPH oxidation and H_2O_2 production in the absence of L-arginine, would corroborate a contributory mechanism of nNOS inhibition involving interaction of the metal with Ca^{2+}/CaM-mediated electron transfer from NADPH to the heme in the oxygenase domain. In this connection, the lack of inhibition of cytochrome *c* reduction in the absence of Ca^{2+}/CaM indicates that the metal does not affect the reductase domain.

Ni^{2+} can bind to CaM at sites other than those specific for Ca^{2+} inducing allostering modifications that can affect the activity of CaM-dependent enzymes (19, 20). Such a mechanism seems to hold also in the present case, since the inhibitory effect of Ni^{2+} was not affected by Ca^{2+}. It is reasonable to hypothesize that Ni^{2+} can convert the Ca^{2+}/CaM complex into a form unable to activate nNOS. The formation of an inactive form of CaM is the mechanism by which melatonin and some kynurenines inhibit nNOS (21). Bromocriptine also inhibits nNOS by a CaM-based mechanism (17). However, Ni^{2+} differs from such CaM-antagonizing inhibitors in that it acts in a competitive manner affecting the L-arginine binding site. In fact, kinetic data indicate that the effect of Ni^{2+} on Ca^{2+}/CaM-dependent activation of nNOS becomes apparent only at low L-arginine concentrations. If the proposed mechanistic frame is valid, the lower effect of Ni^{2+} on iNOS can be explained considering that this isoform is more tightly bound to CaM. However, arguments in the case of eNOS are not so straightforward.

The relevance of the present results to Ni^{2+} toxicity and allergy is an attractive research focus. Mechanisms controlling NOS activity are of particular interest because of the involvement of NO in a broad variety of physiological processes, including vasodilation, neurotransmission and nonspecific immunity (22). In the skin, both constitutive and inducible isoforms of NOS have been identified in a variety of cells, including macrophages, Langerhans cells, dermal fibroblasts, endothelial cells, melanocytes and keratinocytes (23–27), and can be implicated in the control of tissue homeostasis as well as in the modulation of inflammatory processes. Since Ni^{2+}-induced allergic contact dermatitis is considered to be an inflammatory response caused by antigen-specific T cells, it can be speculated that inhibition of cutaneous NOS by the metal plays at least a contributory role in amplifying inflammatory tissue damage by suppression of NO-dependent defense mechanisms.

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