The Effect of Divalent Cations on Neuronal Nitric Oxide Synthase Activity

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INTRODUCTION

Neuronal nitric oxide synthase (NOS I) is a Ca\(^{2+}\)/calmodulin–binding enzyme that generates nitric oxide (NO\(\cdot\)) and L-citrulline from the oxidation of L-arginine, and superoxide (O\(_2\cdot\)\(^-\)) from the one-electron reduction of oxygen (O\(_2\)). Nitric oxide in particular has been implicated in many physiological processes, including vasodilator tone, hypertension, and the development and properties of neuronal function. Unlike Ca\(^{2+}\), which is tightly regulated in the cell, many other divalent cations are unfettered and can compete for the four Ca\(^{2+}\) binding sites on calmodulin. The results presented in this article survey the effects of various divalent metal ions on NOS I–mediated catalysis. As in the case of Ca\(^{2+}\), we demonstrate that Ni\(^{2+}\), Ba\(^{2+}\), and Mn\(^{2+}\) can activate NOS I to metabolize L-arginine to L-citrulline and NO\(\cdot\), and afford O\(_2\cdot\)\(^-\) in the absence of L-arginine. In contrast, Cd\(^{2+}\) did not activate NOS I to produce either NO\(\cdot\) or O\(_2\cdot\)\(^-\), and the combination of Ca\(^{2+}\) and either Cd\(^{2+}\), Ni\(^{2+}\), or Mn\(^{2+}\) inhibited enzyme activity. These interactions may initiate cellular toxicity by negatively affecting NOS I activity through production of NO\(\cdot\), O\(_2\cdot\)\(^-\) and products derived from these free radicals.

Key Words: nitric oxide; superoxide; NOS I; calmodulin; divalent cations; metal toxicity.

A third isoform, inducible NOS (NOS II), is a cytokine-inducible isoform independent of Ca\(^{2+}\) in which calmodulin is permanently bound (Nathan and Xie, 1994). These enzymes are composed of an N-terminal oxidase domain containing an iron protoporphyrin IX (heme), tetrahydrobiopterin (H\(_4\)B) with a binding site for L-arginine, and a C-terminal reductase domain containing the flavin adenine dinucleotide (FAD) and the flavin adenine mononucleotide (FMN). The two domains are connected by a calmodulin-binding motif to which a transient influx of Ca\(^{2+}\) binds (Abu-Soud et al., 1994; Kobayashi et al., 2001; Matsuda and Iyanagi, 1999; Miller et al., 1999). Besides producing NO\(\cdot\), NOS also generates superoxide (O\(_2\cdot\)\(^-\)), the ratio of these free radicals is dependent on the concentration of L-arginine (Pou et al., 1999; Yoneyama et al., 2001). Nitric oxide from NOS I has been implicated in many physiological processes, including vasodilator tone and the development and properties of neuronal function (Moncada and Higgs, 1993; Roskams et al., 1994). In addition, NOS I has been associated with hypertension (Chrissochoidis et al., 2002).

There are regulatory co-factors that dictate whether NOS I generates NO\(\cdot\) and/or O\(_2\cdot\)\(^-\) and H\(_2\)O\(_2\). For instance, in the absence of L-arginine, O\(_2\cdot\)\(^-\) (Pou et al., 1999; Yoneyama et al., 2001) and H\(_2\)O\(_2\) are directly produced; the ratio of these reduction products of O\(_2\) is set by the presence of H\(_4\)B (Rosen et al., 2002). Again, the binding of L-arginine to NOS shifts electron transport from O\(_2\) to this amino acid, producing NO\(\cdot\) at the expense of O\(_2\cdot\)\(^-\) (Pou et al., 1999). Another control element is calmodulin, and, in the presence of Ca\(^{2+}\), it binds to NOS I and enhances electron transport through the reductase domain to the heme, allowing NO\(\cdot\) and O\(_2\cdot\)\(^-\) to be produced [for a review, see Roman et al. (2002)]. We have recently demonstrated that Pb\(^{2+}\) and Sr\(^{2+}\) stimulate the NOS I generation of NO\(\cdot\) and O\(_2\cdot\)\(^-\) by binding to and activating calmodulin (Weaver et al., 2002). Unlike Ca\(^{2+}\), which is tightly regulated in the cell (Clapham, 1995), many divalent cations are unfettered and can compete for the four Ca\(^{2+}\) binding sites on calmodulin. Several other divalent cations such as Ni\(^{2+}\), Ba\(^{2+}\), Cd\(^{2+}\), and Mn\(^{2+}\) have
been shown to bind to and activate calmodulin (Goldstein and Ar, 1983; Habermann et al., 1993; Mills and Johnson, 1985), and several of these divalent cations have been associated with NOS, although the mechanism by which these metals affected NOS is not well understood (Gupta et al., 2000; Mittal et al., 1995; Palumbo et al., 2001; Perry and Marletta, 1998; Yamazaki et al., 1995). Activation of NOS I by divalent cations, other than Ca$_{2+}$, may initiate toxicity through over-production of NO•, O$_2•$-, and products derived from these free radicals.

In the present study, we investigated the possible effects of several divalent metal ions, known to activate calmodulin, on NOS I-mediated catalysis in the absence and presence of L-arginine. We demonstrate that Ni$_{2+}$, Ba$_{2+}$, and Mn$_{2+}$ can activate NOS I to metabolize L-arginine to L-citrulline and NO•. In the absence of L-arginine, these cations also promote NOS I generation of O$_2•$-. It is suggested that these divalent cations, by binding to calmodulin, initiate enzymatic activity. In contrast, Cd$_{2+}$ did not activate NOS I to produce either NO• or O$_2•$-.

**MATERIALS AND METHODS**

**Reagents.** L-arginine, NADPH, calcium chloride, nickel chloride, magnesium chloride, catalase, xanthine oxidase, ferricytochrome c (bovine), hypoxanthine, and calmodulin were obtained from Sigma-Aldrich (St. Louis, MO). Cadmium nitrate was obtained from Allied Chemical (Morristown, NJ). Potassium chloride was obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Manganese chloride, potassium nitrate, and barium chloride were obtained from Fisher Scientific (Fair Lawn, NJ). Superoxide dismutase (SOD) was obtained from Roche Diagnostics (Mannheim, W-Germany). L-[U-14C]arginine monohydrochloride (100 μCi/mmol) was purchased from Amerham Biosciences UK Ltd. (Little Chalfont, Buckinghamshire, UK). 5-tert-Butoxy-carbonyl-5-methyl-1-pyrroline N-oxide (BMPO) was synthesized as described in the literature (Stolze et al., 2003; Tsai et al., 2003). Dowex 50W-X8 cation exchange resin was obtained from Bio-Rad (Hercules, CA). All other chemicals were used as purchased without further purification. Buffers used contained less than 0.01% of inorganic salts, including Ca$_{2+}$.

**Purification of NOS I.** NOS I was expressed and purified essentially as described in the literature (Roman et al., 1995), with the modification that the culture volume was 500 ml rather than 1000 ml. The enzyme concentration was determined by its CO-difference spectrum, as described by Roman et al. (1995), using an extinction coefficient of 100 mM$^{-1}$cm$^{-1}$ at Δε 444–475 nm.

**NOS activation using the [14C]L-citrulline formation assay.** The activation of purified NOS I was determined by its ability to catalyze the formation of L-citrulline from L-arginine as previously reported (Weaver et al., 2002), with modifications. A cocktail solution ([14C]L-arginine (0.6 μCi/ml)) in the presence of NADPH (1 mM), L-arginine (100 μM), and calmodulin (100 μU/ml) in HEPES buffer (50 mM, 0.5 mM EGTA, pH 7.4) was prepared. The reaction was initiated by the addition of the cocktail solution into the reaction mixture containing purified NOS I (3.7 μg) and Ca$_{2+}$, Ni$_{2+}$, Ba$_{2+}$, Mn$_{2+}$, or Cd$_{2+}$ (from 100 μM to 1 mM) to a final volume of 150 μl. The reaction mixture was incubated at room temperature for 10 min and terminated with 2 ml of stop solution (20 mM HEPES, 2 mM EDTA, pH 5.5). The product [14C]L-citrulline was separated by passing the reaction mixture through columns containing Dowex 50W×8 cation exchange resin preactivated with sodium hydroxide (1 M), and radioactivity was counted using a scintillation counter (Model LS 6500; Beckman Coulter Inc., Fullerton, CA). Data were expressed as means and standard deviations of multiple experiments.

**Determination of nitric oxide production.** The initial rate of NO• production by purified NOS I was estimated using the hemoglobin assay (Murphy and Noack, 1994). The reaction was initiated by the addition of NOS I (2.0 μg) to a cuvette containing HEPES buffer (50 mM, 0.5 mM EGTA, pH 7.4), oxyhemoglobin (10 μM), the divalent cation (500 μM), calmodulin (100 U/ml), NADPH (100 μM), and L-arginine (100 μM) to a final volume of 500 μl at room temperature. A UV-Vis spectrophotometer (UVikon, Model 940, Research Instruments International, San Diego, CA) was used to monitor the conversion of oxyhemoglobin to methemoglobin during the course of the reaction. Specifically, the increase in absorbance at 401 nm was used to quantitate the reaction, using an extinction coefficient of 60 mM$^{-1}$cm$^{-1}$ at 401 nm.

**Spin trapping of NOS I-generated O$_2•$- using BMPO.** Spin trapping of O$_2•$- by BMPO was conducted in a reaction mixture containing, NOS I (14.2 μg), various divalent cations (500 μM), and calmodulin (100 U/ml) in phosphate buffer (50 mM, pH 7.4, 1 mM DTPA, 1 mM EGTA). The reaction was initiated by the addition of BMPO (50 mM) and NADPH (100 μM) into the reaction mixture to a final volume of 0.5 ml at room temperature. The reaction mixture was mixed, transferred into a quartz flat cell and fitted into the cavity of the EPR spectrometer. EPR spectra were continuously recorded at room temperature and data shown below in Figure 4 were obtained 10 min after the initiation of the reaction. Instrument settings were as follows: microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 0.5 G; sweep time, 12.5 G/min; and response time, 0.5 s. The receiver gain for each experiment is given in the legend of Figure 4.

**Reduction of ferricytochrome c by NOS I.** The reduction of ferricytochrome c (80 μM) by NOS I (0.4 μg) was monitored in a reaction containing Ca$_{2+}$, Ni$_{2+}$, Ba$_{2+}$, Mn$_{2+}$, or Cd$_{2+}$ (500 μM), calmodulin (100 U/ml), and NOS I in HEPES buffer (50 mM, pH 7.4, 0.5 mM EGTA) at room temperature. The reaction was initiated by the addition of NADPH (100 μM) to the reaction mixture to a final volume of 500 μl. The initial rate of ferricytochrome c reduction in the absence and presence of SOD (30 U/ml) at 550 nm was estimated spectrophotometrically using an extinction coefficient of 21 mM$^{-1}$cm$^{-1}$ at 550 nm (Kathan and Ullrich, 1982).

**RESULTS**

**Effect of Divalent Metal Ions on Calmodulin-Dependent Activation of Purified NOS I**

Several divalent cations have been shown to bind to and activate calmodulin (Goldstein and Ar, 1983; Habermann et al., 1993; Mills and Johnson, 1985). Therefore, we investigated what effect Ba$_{2+}$, Mn$_{2+}$, Ni$_{2+}$, and Cd$_{2+}$, known to affect other calmodulin processes, may have on the activation of calmodulin-dependent NOS I. NOS I activation was estimated by measuring the amount of [14C]L-citrulline produced from L-arginine (100 μM) to which [14C]L-arginine (0.6 μCi/ml) was added in the presence of the different divalent cations, as described in Materials and Methods. Maximal activation for each divalent cation, except for Mn$_{2+}$, was reached at ~500 μM (Fig. 1). Ca$_{2+}$- and Ba$_{2+}$-activated NOS I showed similar dose-response curves,
where in up to 1 mM of each divalent cation salt there was no apparent inhibition in NOS I production of L-citrulline (Fig. 1). Of note, at maximal activation Ba$^{2+}$-activated NOS I was $\sim$70% that of Ca$^{2+}$-activated NOS I. In contrast, NOS I activation by Ni$^{2+}$ and Mn$^{2+}$ exhibited biphasic response curves (Fig. 1) for each metal ion salt, but at concentrations up to 1 mM, NOS I activation decreased by $\sim$90% and $\sim$60%, respectively. At maximal response, Ni$^{2+}$ and Mn$^{2+}$ produced $\sim$40% and $\sim$70%, respectively, of L-citrulline as compared to Ca$^{2+}$-stimulated NOS I (Fig. 1). Interestingly, Cd$^{2+}$ demonstrated negligible NOS I activation ($\sim$9%), even at 500 µM. From these data, it was determined that 500 µM of each divalent cation would produce respective maximal activation under these experimental conditions, and this concentration was used for further experiments.

Next, we examined the effect of various divalent cations on Ca$^{2+}$-activated NOS I. The reactions contained all components described in Materials and Methods, except that the divalent cation, Cd$^{2+}$, Ni$^{2+}$, Ba$^{2+}$, or Mn$^{2+}$ (500 µM), was included along with Ca$^{2+}$ (500 µM). Activation of NOS I was estimated following the formation of [14C]L-citrulline from [14C]L-arginine (0.6 µCi/ml) in the presence of L-arginine (100 µM). The combination of Ca$^{2+}$ and either Cd$^{2+}$, Ni$^{2+}$, or Mn$^{2+}$ resulted in a diminution in enzyme activation by $\sim$90%, $\sim$85%, and $\sim$40%, respectively, as compared to Ca$^{2+}$ alone (Fig. 2). Surprisingly, the combination of Ba$^{2+}$ and Ca$^{2+}$ nearly doubled NOS I response, indicating a synergistic effect of Ba$^{2+}$ on Ca$^{2+}$-activated NOS I.

![FIG. 1. Effect of divalent cations on the formation of L-citrulline by NOS I. The formation of [14C]L-citrulline (represented as % control with respect to Ca$^{2+}$-activated NOS I) from the NOS oxidation of L-arginine. NOS I activity was assayed in the reaction containing NOS I (3.7 µg), NADPH (1 mM), calmodulin (100 U/ml), [14C]L-arginine (0.6 µCi/ml), L-arginine (100 µM) and (●) CaCl$_2$, (▲) BaCl$_2$, (▼) MnCl$_2$, (●) NiCl$_2$, or (○) Cd(NO$_3$)$_2$ (various concentrations) in HEPES buffer (50 mM, 0.5 mM EGTA, pH 7.4). Each point represents the mean ± S.D. from three independent experiments on the same preparation of purified NOS I.](image1)

![FIG. 2. Effect of divalent cations on the activation of NOS I in the presence of Ca$^{2+}$. The formation of [14C]L-citrulline from the NOS oxidation of L-arginine. NOS I activity was assayed in the reaction containing NOS I (3.7 µg), NADPH (1 mM), calmodulin (100 U/ml), [14C]L-arginine (0.6 µCi/ml), L-arginine (100 µM), CaCl$_2$ (500 µM) and either BaCl$_2$ (500 µM), MnCl$_2$ (500 µM), or Cd(NO$_3$)$_2$ (500 µM) in HEPES buffer (50 mM, 0.5 mM EGTA, pH 7.4). Each point represents the mean ± S.D. of the % control from three independent experiments on the same preparation of purified NOS I.](image2)

While the above experiments determined the effect of various divalent cations on NOS-mediated oxidation of L-arginine to L-citrulline and, by implication, NO•, we measured the initial rate of NO• production from NOS I, activated either by Ca$^{2+}$, Ni$^{2+}$, Ba$^{2+}$, Mn$^{2+}$, or Cd$^{2+}$. Initial rates of NO• generation were estimated using the oxyhemoglobin assay, as described in Materials and Methods. For these experiments, the concentration of L-arginine was set at 100 µM, whereas the concentration of Ca$^{2+}$, Ni$^{2+}$, Ba$^{2+}$, Mn$^{2+}$, or Cd$^{2+}$ was fixed at 500 µM. The initial rate of NO• production catalyzed by Ca$^{2+}$ or Ba$^{2+}$ was approximately the same, at $\sim$370 nmoles/min/mg protein (Table 1). In the presence of Cd$^{2+}$, no measurable rate of NO• generation was observed, as compared to the control, in the absence of calmodulin.

A correlation between the initial rates of NO• generation and production of L-citrulline was established by converting the respective values to percent control with respect to Ca$^{2+}$-activated NOS I (Table 1 and Fig. 3). The percent control values show that the initial rates of NO• production are comparable to the L-citrulline assay, each following the same trend of activation in which Mn$^{2+}$ and Ni$^{2+}$ produced the least amount of NO•.

Control experiments were also performed to ensure that the counter ions, Cl⁻ or NO₃⁻, did not contribute to the observed results (Nishimura et al., 1999; Schrammel et al., 1998). Substituting KCl (500 µM) or KNO$_3$ (500 µM) for the divalent salts resulted in no activation of NOS I in the absence of Ca$^{2+}$ or inhibition in the presence of Ca$^{2+}$ (500 µM) (data not shown). It was concluded that any effect these counter ions have on NOS I activity is negligible in this study, and all observations were a
consequence of the respective divalent cations. These data also further confirmed that Ca\(^{2+}\) does not activate NOS I.

### Spin Trapping of NOS-Generated O\(_2\)•⁻

NOS I produces O\(_2\)•⁻ in the absence of L-arginine (Pou et al., 1992; Yoneyama et al., 2001). Of interest is whether Ba\(^{2+}\), Ni\(^{2+}\), or Mn\(^{2+}\), which activate NOS I to metabolize L-arginine to L-citrulline and NO•, can, in the absence of substrate, generate O\(_2\)•⁻. Given that ferricytochrome c and its derivatives cannot report NOS generated O\(_2\)•⁻ (Weaver et al., 2003), we turned to spin trapping/EPR spectroscopy. For these experiments, BMPO was chosen as the spin trap, because its reaction with O\(_2\)•⁻ gives a spin-trapped adduct, BMPO-OOH, that exhibits a long half-life (Rosen et al., 2002). As depicted in Figure 4, BMPO spin trapped O\(_2\)•⁻ from NOS I activated by various divalent cations. However, the EPR spectral peak height of BMPO-OOH was much greater with Ca\(^{2+}\) than with Ba\(^{2+}\), Ni\(^{2+}\), and Mn\(^{2+}\) (500 μM for each ion)-activated NOS I. These data suggested that NOS I generated considerably less O\(_2\)•⁻ when activated by these metal ions than when activated by Ca\(^{2+}\). In the case of Mn\(^{2+}\), the EPR spectral peak height of BMPO-OOH was diminished by nearly 80% as compared to control (Fig. 4).

### The Effect of Divalent Cations on the Rate of Ferricytochrome c Reduction by O\(_2\)•⁻

To determine whether the decrease in O\(_2\)•⁻ spin trapping was attributable to competition for O\(_2\)•⁻ by Ba\(^{2+}\), Ni\(^{2+}\), or Mn\(^{2+}\), a series of control experiments were performed. A hypoxanthine/xanthine oxidase system was used as a source of O\(_2\)•⁻. And the SOD-like property of each of the divalent cations (500 μM), which was dissolved in the buffer used in the experiments above, was estimated by monitoring the initial rate of ferricytochrome c reduction as compared to that observed with SOD (30 U/ml). The control was performed in the absence of these metal cations. In the absence of divalent cations, 0.613 ± 0.17 μM/min of O\(_2\)•⁻ was generated, as measured by the reduction of ferricytochrome c, and in the presence of Ca\(^{2+}\), Ba\(^{2+}\), Ni\(^{2+}\), or Mn\(^{2+}\), 0.598 ± 0.17, 0.547 ± 0.18, 0.604 ± 0.15, or 0.540 ± 0.16 μM/min of O\(_2\)•⁻, respectively, was observed. From these data, no significant SOD-like property was attributed to any of the divalent cations.

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**TABLE 1**

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<tr>
<th>Metal salts (500 μM)</th>
<th>NO• production (nmoles/min/mg protein)</th>
<th>% Control</th>
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<tr>
<td>CaCl(_2)</td>
<td>387 ± 28</td>
<td>100 ± 7.3</td>
</tr>
<tr>
<td>BaCl(_2)</td>
<td>364 ± 10</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>MnCl(_2)</td>
<td>290 ± 47</td>
<td>75 ± 10</td>
</tr>
<tr>
<td>NiCl(_2)</td>
<td>217 ± 36</td>
<td>55 ± 9</td>
</tr>
<tr>
<td>Cd(NO(_3))(_2)</td>
<td>3.32 ± 6.0</td>
<td>0.8 ± 2</td>
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* Rates are the average of three independent experiments, expressed as the means and standard deviations.
Reduction of Ferricytochrome c by Divalent Cation–Activated NOS I

As reported in the literature, ferricytochrome c is reduced by NOS I (Heinzel et al., 1992; Mayer et al., 1991; Pou et al., 1992; Roman et al., 2000; Sheta et al., 1994). This enzymatic reduction occurs in the absence and presence of calmodulin, although the reduction in the absence of calmodulin is 10-fold less than in the presence of calmodulin when NOS I is activated using Ca$^{2+}$/calmodulin (Roman et al., 2000). Therefore, experiments were conducted to determine what effect Ba$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, and Cd$^{2+}$ had on the rate of NOS I reduction of ferricytochrome c (Table 2). Each divalent cation–activated NOS I, except Cd$^{2+}$, was able to reduce ferricytochrome c at a rate comparable to that of Ca$^{2+}$. Also, each cation-activated NOS I followed the trend of maximal activation similar to that seen in previous experiments. Of note, the rates of ferricytochrome c reduction in the presence of Cd$^{2+}$-activated NOS I, with and without calmodulin, were the same, confirming that the activation of NOS I as measured by the L-citrulline assay by Cd$^{2+}$ is negligible. In each case, whether calmodulin was present or not, SOD (30 U/ml) did not inhibit the reduction of ferricytochrome c. These data demonstrate that this reduction was not a consequence of O$_2$••, but resulted from direct reduction by NOS I, as shown previously (Pou et al., 1992).

### Discussion

Activation of calmodulin occurs when Ca$^{2+}$ occupies all four EF-hand domains (Kern et al., 2000). The binding of Ca$^{2+}$ to calmodulin produces a conformational change that converts the protein to an active form, which binds to NOS I, allowing electron transport through the reductase domain of NOS to the oxidase domain to facilitate the production of NO•, O$_2$••, and H$_2$O$_2$. Several divalent and trivalent cations besides Ca$^{2+}$ can bind to calmodulin and exert cellular toxicity by altering the normal homeostasis of the Ca$^{2+}$/calmodulin pathway (Habermann et al., 1993; Mills and Johnson, 1985; Ozawa et al., 1999). We explored the effect Ni$^{2+}$, Ba$^{2+}$, Cd$^{2+}$, or Mn$^{2+}$ has on calmodulin-dependent NOS I-generated NO• and O$_2$••.

The results described in this article indicate that Ni$^{2+}$, Ba$^{2+}$, and Mn$^{2+}$ can activate NOS I, producing NO•, and O$_2$•• (Figs. 1 and 3 and Table 1). In the presence of Ca$^{2+}$, formation of L-citrulline from L-arginine was inhibited with the inclusion of Ni$^{2+}$, Cd$^{2+}$, or Mn$^{2+}$ (Fig. 2). The increase in L-citrulline formation seen with the simultaneous inclusion of Ca$^{2+}$ and Ba$^{2+}$ was surprising. We are exploring this synergistic phenomenon in more detail. Of note, although the trend of activation is the same, the percent control values for NO• generation and L-citrulline production differed by ~10% for Ni$^{2+}$ and Mn$^{2+}$, with the values for Ba$^{2+}$ differing by ~20%. Because the trend of activation remained the same independent of the assay, these differences were disregarded.

In the absence of L-arginine, each divalent cation–activated NOS I was shown to reduce ferricytochrome c at rates comparable to Ca$^{2+}$-activated NOS I (Table 2). Similarly, ferricytochrome c reduction by NOS I in the absence of calmodulin was ~10-fold less than in its presence (Table 2), confirming an earlier report (Roman et al., 2000). This finding supports the notion that the activity associated with these divalent cations is a result of these metal ions binding to calmodulin. Depending on the divalent cation used to activate NOS, differing amounts of O$_2$•• were generated. This observation was similar to that seen with L-citrulline and NO• production (Figs. 3 and 4). However, the disparity in NO• production of O$_2$••, NO• cannot be overlooked. Several control experiments verified that the disparity in the EPR spectral peak heights correlated with variable amounts of NO•-generated O$_2$••, and that these differing EPR spectral peak heights were not attributed to other reactions taking place in the reaction mixture, such as variable SOD-like activity of the metal ions or an enhanced rate of BMPO-OOH decomposition of the BMPO-OOH in the presence of the various divalent cations.

We offer several possible explanations for the differences in L-citrulline, NO•, and O$_2$•• production by these metal ions. It has been suggested that cations with a specific range of radii are required to induce the necessary conformational change for calmodulin activity (Ozawa et al., 1999). Therefore, a plot of NOS I activation versus ionic radii was developed (Fig. 3). This plot confirms that the initial rates of NO• generation and total L-citrulline production follow the same trend, and that a specific range of radii is necessary for maximal activation with calmodulin. Divalent cations smaller or larger than Ca$^{2+}$ exhibit decreases in activation. In contrast, no activation was observed using Cd$^{2+}$ with ionic radii of 1.09 Å (Huheey et al., 1993), and previous work observed that Sr$^{2+}$-activated NOS I was similar to Ca$^{2+}$-activated NOS I, although Sr$^{2+}$ (1.32 Å) has a similar ionic radius to Pb$^{2+}$ (1.33 Å), which showed minimal activation, (Huheey et al., 1993; Weaver et al., 2002). These findings suggest that other factors are involved for calmodulin activation of NOS I. In addition, Cd$^{2+}$ has been shown to activate other calmodulin-dependent processes, although NOS I activation was not observed in these studies (Habermann et al., 1993; Ozawa et al., 1999).

### Table 2

<table>
<thead>
<tr>
<th>Metal salts (500 μM)</th>
<th>Reduction rate (μM/min/μg protein)*</th>
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<tr>
<td></td>
<td>With calmodulin</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>43.1 ± 6.2</td>
</tr>
<tr>
<td>NiCl$_2$</td>
<td>29.3 ± 5.0</td>
</tr>
<tr>
<td>BaCl$_2$</td>
<td>37.5 ± 8.4</td>
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<tr>
<td>MnCl$_2$</td>
<td>38.8 ± 5.2</td>
</tr>
<tr>
<td>Cd(NO$_3$)$_2$</td>
<td>2.35 ± 0.80</td>
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*Rates are the average of three independent experiments, expressed as the means and standard deviations.

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A review of hard-soft acid-base (HSAB) concepts may also provide insight into the activation of NOS I by these various divalent cations (Huheey et al., 1993). Metal ions most frequently bind to donor ligands according to preferences dictated by HSAB. Mainly, hard metals prefer hard ligands, and soft metals prefer soft ligands. Calmodulin binds to Ca$^{2+}$ via side-chain carboxylates, alcohol, or carboxamide oxygen, or via backbone carbonyl groups (Lippard and Berg 1994). These ligands are classified as hard ligands and prefer to bind to hard metals such as Ca$^{2+}$, Ba$^{2+}$, and Mn$^{2+}$, and they do not bind favorably with Ni$^{2+}$, classified as a soft metal. The decrease in NOS I activation by Ni$^{2+}$ supports this theory, whereas Mn$^{2+}$ and Ba$^{2+}$ exhibited near maximal activation compared to Ca$^{2+}$. However, the biphasic curves observed with Mn$^{2+}$ and Ni$^{2+}$ suggest that still other factors are involved.

Other studies have supported our observations in which divalent cations can promote or inhibit NOS activity from homogenate (Gupta et al., 2000; Mittal et al., 1995; Yamazaki et al., 1995), purified (Perry and Marletta, 1998), or recombinant (Palumbo et al., 2001) NOSs as well as NO• generated from macrophages (Tian and Lawrence, 1996). For instance, Ni$^{2+}$ has also been shown to enhance NOS I activity in the brain and adrenal glands (Gupta et al., 2000), whereas it has been shown to inhibit NOS I activity in other studies (Mittal et al., 1995; Palumbo et al., 2001). Several mechanisms have been suggested for this activation and inhibition of NOS (Gupta et al., 2000; Palumbo et al., 2001), but the results shown here suggest that Ni$^{2+}$ and other divalent cations can bind to calmodulin in a manner similar to Ca$^{2+}$, thereby activating NOS directly. This finding appears to be concentration specific, as higher concentrations of several metals ions did not promote activation (Fig. 1). At higher concentrations of these divalent cations, further alterations in calmodulin conformation may prevent further activation. Our results are also in agreement with studies that show that high concentrations of specific metals ions in the presence of Ca$^{2+}$ promote NOS inhibition (Mittal et al., 1995; Palumbo et al., 2001), possibly by further altering calmodulin, as mentioned above. Cd$^{2+}$ appears not to produce the desired conformational change of calmodulin for NOS activation in the absence of Ca$^{2+}$, and it may only promote changes in calmodulin not suitable for NOS activation, as only inhibition was observed.

As described by Gupta et al. (2000), Ni$^{2+}$ can be accumulated in the brain after exposure. Although it is unclear whether the concentration accumulated in the brain would be sufficient to apply to our results, it is noteworthy. Among the many toxic effects of Ni$^{2+}$ in biological systems is the ability of this divalent cation to affect Ca$^{2+}$ homeostasis, produce low but measurable levels of other free radicals, and participate with NO• in Ni$^{2+}$-induced hyperglycemia (Denkhaus and Salnikow, 2002; Gupta et al., 2000). Also, NOS I is present in non-neuronal cell types in addition to the brain and other neuronal cell types (for a review, see Försterrmann et al. (1998)). Accumulation of Ni$^{2+}$ or other divalent cations in such tissues may elicit the response observed in this study. Of note, these results reflect stimulation or inhibition from the total concentration of metal salts added. The free ion concentrations would need to be determined to correlate these findings to possible concentrations of these metal ions in vivo.

The toxic effects of Ba$^{2+}$ include gastrointestinal, cardiovascular, respiratory, and neuromuscular symptoms, and it directly affects vascular, cardiac, and gastrointestinal smooth muscle (Jacobs et al., 2002). Hypertension has also been reported in both laboratory and clinical studies of Ba$^{2+}$ (Jacobs et al., 2002). Excessive Mn$^{2+}$ intake can result in neurobehavioral deficits, neurological syndrome similar to chronic Parkinson’s disease, and oxidative stress (Chetty et al., 2001; Husain et al., 2001). Cadmium has been associated with hypertension and myocarditis among its many toxic effects (Kopp et al., 1983; Thun et al., 1989). We suggest that toxicity related to Cd$^{2+}$ is the result of its ability to inhibit NOS I catalysis, whereas other metal ions may exert toxicity by either producing or inhibiting free radical production dependent on the concentration of metal ion present.

Results reported here should be interpreted with caution because the free ion concentration under the conditions presented is not known. It also cannot be stated that these divalent cations reach concentrations sufficient for calmodulin activation in biological systems as described in this paper. These results merely suggest a model by which several divalent cations may affect NOS I activity. Because NO• is involved in many physiological processes (Moncada and Higgs, 1993), toxic effects could be attributed to the ability of these divalent cations to affect NOS I activity as demonstrated in this paper.

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