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Cytotoxicity by Lead-induced nNOS Phosphorylation in a Dopaminergic CATH.a Cells: Roles of Protein Kinase A

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Abstract

Neuronal cell toxicity induced by decreased nitric oxide (NO) production may be caused by modulation of constitutive neuronal NO synthase (nNOS). We used lead acetate (Pb^{2+}) to modulate physiological NO release and the related pathways of protein kinases like PKC, CaM-KII, and PKA in CATH.a cells, a dopaminergic cell line that has constitutive nNOS activity. In the cells treated with Pb^{2+}, cell viability and modulation (phosphorylation) levels of nNOS were determined by MTT assay and Western blot analysis, respectively. nNOS reductase activity (cytochrome *c*) was also assessed to compare the phosphorylation site-specific nNOS activity. nNOS activity was also determined by NADPH consumption rates. Pb^{2+} treatment alone increased the phosphorylation of nNOS with decreased reductase activity. The phosphorylation levels increased markedly with decreased nNOS reductase activity, when Pb^{2+} was combined with inhibitors for two (PKC and CaM-KII) or three (PKA, PKC and CaM-KII) protein kinases. Interestingly, when the cells were exposed to Pb^{2+} plus PKC or CaM-KII inhibitor, the nNOS was phosphorylated strongly with the lowest activity. However, the levels of phosphorylated nNOS following Pb^{2+} treatment decreased significantly after combined treatment with the PKA inhibitor, and Pb^{2+}-induced suppression of reductase activity did not occur. These results demonstrate that physiological NO release in the neuronal cells exposed to Pb^{2+} can be decreased by PKA-mediated nNOS phosphorylation that may be caused by interactions with PKC and/or CaM-KII.

Keywords: Lead, Cytotoxicity, CATH.a cells, Nitric oxide, nNOS phosphorylation, PKA

Lead (Pb^{2+}) is a potent environmental toxicant that affects the nervous system, resulting in mental retardation, impaired cognitive function, and behavioral abnormalities, particularly in children[1,2]. A number of studies have demonstrated that Pb^{2+}-induced cytotoxicity may occur in almost all of the classical neurotransmitter systems, including dopaminergic, cholinergic, GABAergic, and glutamatergic[3,4]. However, the neurobiological mechanisms of Pb^{2+}-induced cytotoxicity are still not understood.

Pb^{2+} alters intracellular calcium homeostasis, and thus Ca^{2+}-mediated processes in cellular pathways may be altered, particularly in systems mediated by phosphorylation. In general, PKC activation is modulated in a Ca^{2+}-dependent manner. However, most common mechanisms mediated by intracellular calcium are controlled via the activation of Ca^{2+}/ calmodulin-dependent CaM-kinase. CaM-kinase II phosphorylates itself, as well as other cell proteins, in response to increased Ca^{2+}/calmodulin, which is thought to be a major mechanism for maintaining kinase activity. Furthermore, CaM-kinase II can be activated by low concentrations of Pb^{2+}.

Other studies have demonstrated that PKC inhibits the activity of neuronal nitric oxide (nNOS) by direct phosphorylation of the enzyme[5,6]. Similarly, regulation of nNOS by calmodulin kinase has been reported in neuronal cells[7]. This indicates that nNOS activity may be affected by Ca^{2+}-dependent kinase activation mediated by Pb^{2+}, either directly or indirectly.

NO generated endogenously by nNOS plays a crucial role in synaptic plasticity in the central nervous system (CNS)[8,9]. nNOS has been shown to be phosphorylated by cyclic AMP-dependent protein kinase (PKA), cyclic GMP-dependent protein kinase and Ca^{2+}-dependent protein kinases (PKC and CaM kinases), with each kinase phosphorylating a different serine site on the NOS[10-13]. Currently, phosphorylat-

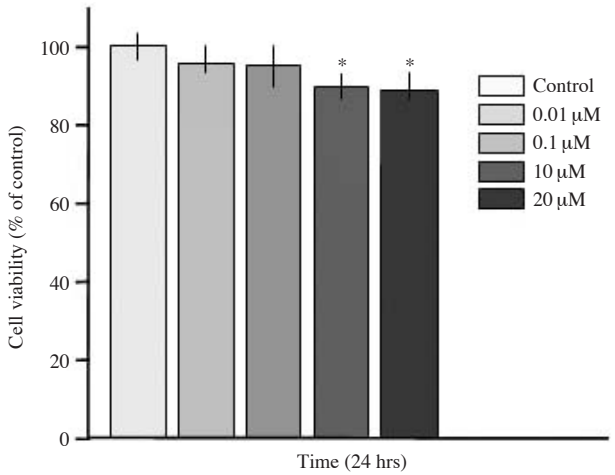

Figure 1. Effect of Pb^{2+} on cell viability determined in CATH.a cells treated time-dependently with four different concentrations. Cell viability is expressed as mean percentages (±SE) over control values from six independent experiments. Statistically significant decreases from the control are indicated as $*P<0.05$.

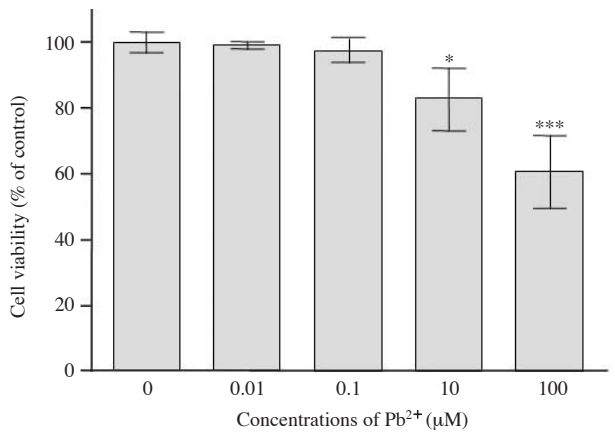

Figure 2. Effect of Pb^{2+} on cell viability determined in CATH.a cells treated with four different concentrations for 24 hrs. Cell viability is expressed as mean percentages (±SE) over control values from six independent experiments. Statistically significant decreases from the control are indicated as $*P<0.05$ and $***P<0.001$.

ion of nNOS is considered an inhibitory mechanism for NO production. In general, insufficient NO production is a pathologic mediator that leads to cytotoxicity. Thus, disruption of physiological NO production by protein kinases may lead to cytotoxicity and result in cellular damage.

Therefore, in the present study, we investigate whether neuronal cytotoxicity induced by Pb^{2+} is mediated by modulation in NO release mediated by

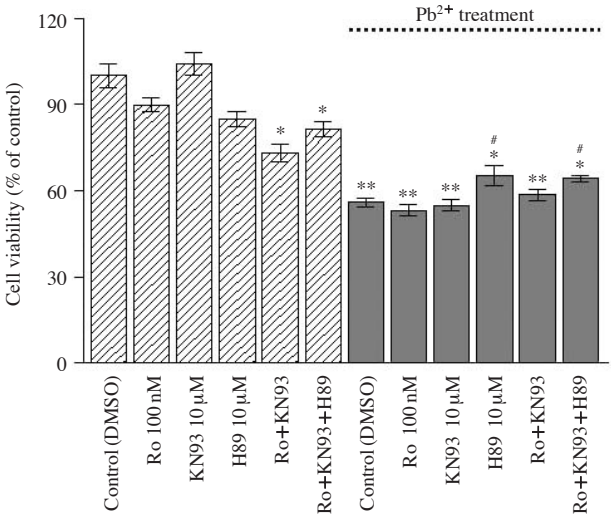

Figure 3. Effect of single or combined protein kinase inhibitors on cell viability in the absence or presence of 100 μM Pb^{2+}. Cell viability is expressed as mean percentages (±SE) over control and each Pb^{2+} untreated values from four independent experiments. Statistically significant decreases or increase from the control are indicated as $*P<0.05$, $**P<0.01$ and $^{\#}P<0.05$.

interactions between protein kinases and nNOS.

Pb^{2+}-mediated Cytotoxicity of Neuronal Cells

To determine the effect of Pb^{2+} on cell viability, CATH.a cells were treated with various concentrations of Pb^{2+} (0.01, 0.1, 1, 10 and 20 μM) for 2, 8, 16 and 24 hrs. For all concentrations of Pb^{2+}, the viability of the cells treated for 24 hrs only decreased (Figure 1). As shown in Figure 2, thus, for cells exposed to 0.01, 1, 10 and 100 μM of Pb^{2+} for 24 hrs, the percentages of viable cells were 98.4±1.2, 97±3.8, 82.5±9.8 and 60.5±11% of total, respectively.

Effects of Inhibition of Protein Kinases on the Viability of Pb^{2+}-treated and -untreated Cells

To determine whether the specific inhibition of protein kinase pathways offers protection from Pb^{2+}-induced cytotoxicity, CATH.a cells were pre-treated before 10 min with each protein kinase inhibitor. Cell viability did not change after treatment with single dose of inhibitors, as shown in Figure 3, but decreased significantly in the samples treated with two (Ro-31-8220+KN-93) or three inhibitors (Ro-31-8220+KN-93+H-89) (73.1±6.4% or 81.4±5.1%, respectively, $*P<0.05$). Pb^{2+} treatment markedly decreased the cell viability to 56.4±3.2% compared to untreated controls. Furthermore, when cells pre-treated with

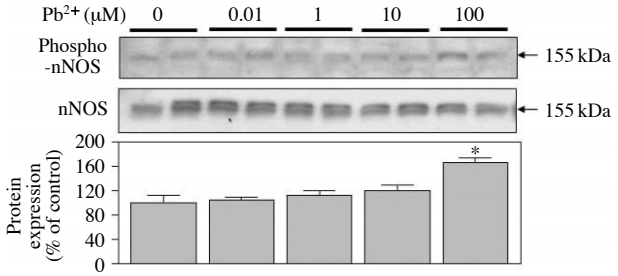

Figure 4. Effect of Pb^{2+} on nNOS and phosphorylated nNOS expression determined by Western-blot analysis in CATH.a cells treated with four different concentrations. Band intensify analyzed densitometically is expressed as mean percentages (±SE) over control values from four independent culture cells. Statistically significant increase from the control is indicated as $*P<0.05$.

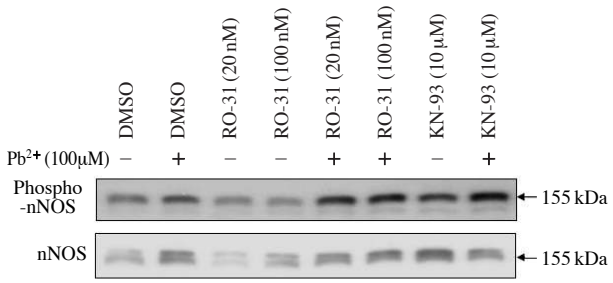

Figure 5. Effect of single protein kinase inhibitor with different concentrations on the phosphorylated nNOS and nNOS expression in the absence or presence of 100 μM Pb^{2+}. Band intensity analyzed densitometrically is expressed as mean percentages (±SE) over control values from four independent samples. Statistically significant decrease or increase from the control are indicated as $**P<0.01$ and $^{\#}P<0.05$, respectively.

Ro-31-8220, KN-93, or Ro-31-8220 plus KN-93 were exposed to Pb^{2+}, the cell viability also decreased to levels similar to those for cells treated with Pb^{2+} only (53.3±3.9%, 54.9±4.0% and 58.6±4.0%, respectively, $**P<0.01$). However, when cells pretreated with H-89 only, or three inhibitors including H-89 were exposed to Pb^{2+}, the cells were partially protected from death, compared with cells only treated with Pb^{2+} (65.0±7.1% or 64.3±2.2%, respectively, $^{\#}P<0.05$), although the cell viability decreased significantly compared with the controls ($*P<0.05$, Figure 3).

Effects of Pb^{2+} on nNOS Expression and Its Phosphorylation

In the Western-blot analysis, the nNOS expressed in CATH.a cells was the same size as that found in a rat brain (approximately 155 kDa). The levels of nNOS expression and phosphorylation detected did not change significantly in cells exposed to variable concentrations of Pb^{2+} (Figure 4). However the phosphorylated nNOS levels increased significantly after treatment with 100 μM Pb^{2+} (165±8.2% of controls) ($*P<0.05$, Figure 4).

Effects of Inhibition of Protein Kinases on nNOS Expression

To determine phosphorylation of nNOS yielded by Pb^{2+} blocked via each protein kinase inhibitor. In CATH.a cells treated with Pb^{2+}, the level of phosphorylated nNOS increased to 120.5±10.0%, compared with control cells treated with DMSO, as shown in Figure 5, but decreased slightly in the samples treated with 20 nM of Ro 31-8220 or 100 nM of Ro 31-8220 (73.1±6.4 and 81.4±5.1, respectively, $**P<0.01$). Interestingly, when the cells were treated with RO-31 or KN-93, the levels of phosphorylated nNOS increased (Figure 5).

Effects of Inhibition of Protein Kinases on the Catalytic Activity of nNOS

Levels of phosphorylated nNOS increased significantly in cells treated with Pb^{2+} plus the PKC (Ro-31-8220) and/or CaM-kinase II inhibitors (KN-93). In this study, it is important to determine if phosphorylation of nNOS alters the catalytic activity of nNOS. We have assessed catalytic activity using a NADPH oxidation assay (Figure 6). In cells exposed to 100 μM of Pb^{2+}, the catalytic activity of nNOS decreased significantly to approximately 36.7±10.0% compared with untreated controls. The catalytic activities of nNOS after treatment with specific inhibitors of protein kinases (Ro-31-8220, H-89, Ro-31-8220+KN-93 and Ro-31-8220+KN-93+H-89) were slightly decreased without significance (203.3±30.5, 178.2±7.39, 191.1±48.1 and 172.4±37.4 nmol/min/mg, respectively), but decreased significantly to 72.4±3.4% (159.1±12.9 nmole/min/mg) in cells treated with CaM-kinase II inhibitor. However, when cells were treated with Pb^{2+} in addition to each protein kinase inhibitor (Ro 31-8220 and KN-93) except for the PKA inhibitor (H-89), the catalytic activities of nNOS were inhibited to levels similar to those for cells only treated with Pb^{2+} (97.1±59.5 and 87.9±13.1 nmol/min/mg, respectively, $**P<0.01$). However, when Pb^{2+} was combined with H-89, the two or three inhibitors together, nNOS catalytic activities were particularly restored (138.1±31.0, 121.5±25.4 or 132.8±15.6 nmol/min/mg, respectively, $^{\#}P<0.05$) compared with samples from cells treated with Pb^{2+} only (85.1±24.4 nmol/min/mg) (Figure 6).

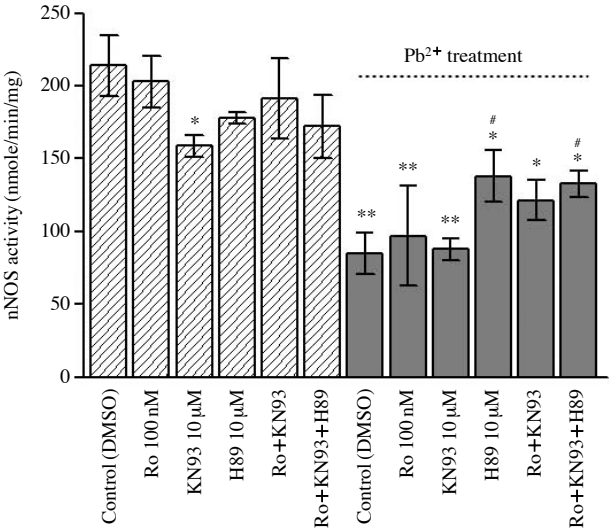

Figure 6. Effect of single and combined protein kinase inhibitor on nNOS activities in the cells exposed to 100 μM of Pb^{2+}. nNOS activity was protected in used PKA inhibitor (H89). Statistically significant decrease or protection from the control and the reduction of activity are indicated as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{\#}P < 0.05$, respectively.

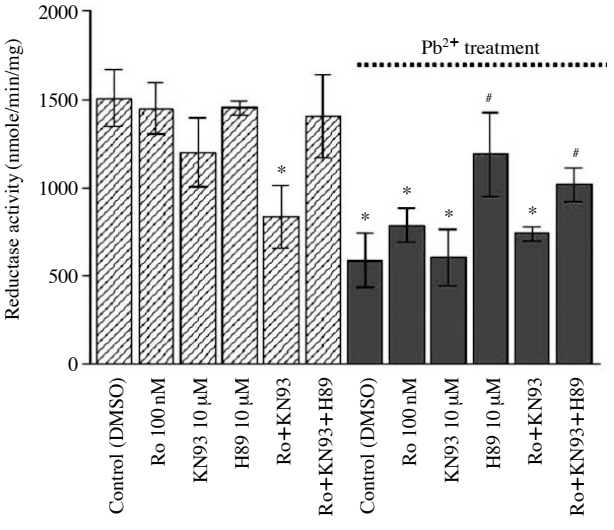

Figure 7. Effect of single and combined protein kinase inhibitor on nNOS-mediated reductase activities in the absence or presence of Pb^{2+}. nNOS-catalyzed reduction of cytochrome *c* was significantly decreased after co-treatment of Pb^{2+}. However, the decrease of reductase activity was protected in used PKA inhibitor. Statistically significant decrease or protection from the control and the reduction of activity are indicated as $^{*}P < 0.05$ and $^{\#}P < 0.05$, respectively.

Effects of Inhibition of Protein Kinases on the Reductase Activity of nNOS

All NOS isoforms catalyze electron transfer to a variety of electron acceptors including cytochrome *c* and DCPI. To determine how the reductase activity of nNOS was altered through inhibition of the protein kinase pathways, we assessed reductase activity in the presence (cytochrome *c*) or absence (DCPI) of Ca^{2+}/Calmodulin.

In cells exposed to 100 μM of Pb^{2+}, phosphorylated nNOS exhibited Ca^{2+}-dependent reduction of cytochrome *c*, representing approximately 62.0% of untreated control enzyme. The reductase activities of nNOS after treatment with specific inhibitors of protein kinases (100 nM of Ro-31-8220, 10 μM of KN-93, 10 μM of H-89 or 100 nM of Ro-31-8220+10 μM of KN-93+10 μM of H-89) was similar to that for untreated controls (data not shown). However the reductase activity of nNOS decreased in cells treated with two inhibitors (100 nM of Ro-31-8220+10 μM of KN-93), as show in Figure 7 (833.3 ± 309.5 nmol/min/mg, $^{*}P < 0.05$). Interestingly, when Pb^{2+} was co-treated with Ro-31-8220, KN-93 or Ro-31-8220+KN-93, the reductase activity of nNOS was inhibited to similar levels to that for cells only treated with Pb^{2+} (785.7 ± 166.7, 602.8 ± 279.7 or 738.1 ± 71.4 nmol/min/mg, respectively, $^{*}P < 0.05$). However, when Pb^{2+} was co-treated with only H-89 or with the three inhibitors including H-89, activity increased dramatically (1190.0 ± 415.1 or 1015.0 ± 167.2 nmole/min/mg, respectively) compared with the sample treated with Pb^{2+} only (586.6 ± 270.5 nmole/min/mg, respectively, $^{\#}P < 0.05$) (Figure 7).

Discussion

In the present study, we observed that a high concentration of Pb^{2+} can modulate neuronal NO synthase (nNOS) through activation of the protein kinases PKA, PKC and CaM-KII, leading to a decrease in physiological NO concentration and neuronal cytotoxicity.

In general, it has been shown that a low concentration of Pb^{2+} (< 1 μM) leads to the activation of PKC, thereby increasing intracellular free calcium, whereas high concentrations of Pb^{2+} (> 1 μM) may lead to cell death by decreasing PKC and subsequent free calcium levels[14]. These results indicate that Pb^{2+}, depending on its concentration, may either activate or inhibit PKC. Thus, PKC activity may be modulated in the neuronal pathways.

In the central nervous system, constitutively-expressed nNOS controls the physiological roles of NO. However, NO, a neurotransmitter can be released differentially by modulation of nNOS gene expression or activity. Changes in nNOS gene expression, at the

mRNA or/and protein levels, have been reported in neurological diseases such as Parkinson's disease[15]. However, there are few reports of the modulation of nNOS protein activity through post-translational modifications. Recently, Hayashi, Y. *et al*. has reported that nNOS enzyme activities decreased following CaM kinase-induced phosphorylation at Ser 847, resulting in a decreased physiological NO release[7]. Dephosphorylation of nNOS at Ser 847 is mediated by phosphatase-2A[16].

nNOS exists extensively in the central nervous system (CNS) and its activity can be modulated by PKA-, CaM-KII- and/or PKC-mediated phosphorylation. Those protein kinases phosphorylate different serine sites on nNOS, resulting in the inhibition of nNOS activity[10-13].

Physiological regulation of NO by nNOS is more important than other conventional regulatory mechanisms, because NO cannot be stored, released, or rendered inactive after synaptic release. NO deficiency is particularly implicated in neuronal cell damage in cells producing NO through NOS activity. Recently it has been suggested that decrease in NO production induces the cytotoxicity seen in CATH.a cells treated with Aroclor 1254[17]. Speculatively, this data may indicate that reduction in NO production by protein kinases can also control cell viability.

We found that high concentrations of Pb^{2+} significantly decreased cell viability in dopaminergic neuronal cells that release NO spontaneously. Furthermore, Pb^{2+} treatment increased the levels of nNOS phosphorylation, decreasing its reductase activity. High concentrations of Pb^{2+} brought about activation of protein kinases and thus increased phosphorylation of nNOS and decreased nNOS activity. Jadhav, A.L. *et al*. report that the PKC inhibitor staurosporine (100 nM) partially blocked cell death in PC12 cells treated with low concentrations (Pb^{2+})[14]. By contrast, in our study, inhibition of PKC, CaM-KII or PKC plus CaM -KII pathways did not decrease the phosphorylation levels of nNOS. Interestingly, a PKA inhibitor significantly decreased phosphorylation of nNOS, and protected the cells against Pb^{2+}-induced cytotoxicity. These results indicate that cytotoxicity caused by high concentrations of Pb^{2+} may be caused by partial or complete blockage of PKA pathway in CATH.a cells. In turn, cytotoxicity is partially blocked through inhibition of the protein kinase A pathway.

In general, neuronal cell toxicity induced by overproduction of NO may be caused by peroxynitrite produced in reactions between NO and superoxide anion formed by NADPH oxidation[18-20]. There are few reports of neuronal cytotoxicity caused by low levels of NO production due to down-regulation of nNOS or its modification. However, in this study, we found that decreasing NO by modulation of protein kinases resulted in neuronal cytotoxicity.

In conclusion, these results demonstrate that physiological NO production in dopaminergic neuronal cells is reduced by nNOS phosphorylation, mediated by the PKA pathway, and with possible interactions with PKC and/or CaM-KII. Thus, Pb^{2+}-induced cytotoxicity of neuronal cells may be induced by modulation of NO production.

Methods

Chemicals

Lead acetate (Pb^{2+}), MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), 2, 6-Dichloroindophenol (DCPI), Cytochrome c, and triethanolamine were purchased from Sigma (St. Louis, MO). PKC inhibitor (RO 31-8221), CaM-kinase II (KN-93), and PKA inhibitor (H-89) were purchased from Calbiochem (La Jolla, CA). RPMI 1640 medium, Fetal Bovine Serum (FBS), Horse Serum (HS) and streptomycin/penicillin were obtained from Gibco BRL (Gaithersburg, MD). Immunological reagents were from Amersham (Aylesbury, UK). Anti-nNOS antibody was from Transduction Laboratories (Lexington, KY). The anti-phosphorylated-nNOS antibody was produced using the specific synthetic peptide [CKVRFNS (phosphoS) VSSYS]. 8-16% Tris -Glycine gradient gels were from Novex (San Diego, CA).

Cell Culture

CATH.a cells, originally produced by Dr Chikaraishi, were donated by Tufts University Medical School[21]. CATH.a cells are a dopaminergic cell line derived from a brain tumor from transgenic mice carrying the SV40 T-antigen under the transcriptional control of the rat tyrosine hydroxylase gene. CATH.a cells contain the catecholamine biosynthetic enzymes and produce both dopamine and norepinephrine in a ratio of 5 : 1 and constitutively produce NO[21,22]. Cells were grown in RPMI 1640 medium containing 8% HS, 4% FBS, 100 IU/L penicillin and 10 μg/mL streptomycin, at 37°C, in a humidified atmosphere of 95% air and 5% CO_2. In the experiments, cells were plated in tissue culture dishes at a density of 2×10^4 cells/well in 96-well culture plates or 5×10^6 cells per 100-mm plate.

Protein Extraction

CATH.a cells were plated on tissue culture dishes at a density of 5×10^6 cells per 100-mm plate. After

24 hrs, cells were fed with 5 mL of serum-free media, at which time protein kinase C inhibitor, protein kinase A inhibitor and CaM kinase II inhibitor were added to each plate. After 10 minutes, 5 mL Pb^{2+} (200 μM) in serum-free media was added, and the cells were incubated for 24 hrs. Cells were then washed with 1 × PBS, scraped into 120 μL of lysis buffer containing 320 mM sucrose, 200 mM HEPES, 1 mM EDTA, with proteinase inhibitors, and sonicated on ice. The crude extracts were centrifuged at 500 × g for 5 minutes and the supernatants, containing the total cellular protein, were mixed with glycerol to a final concentration of 20% of total volume, and stored at −80°C. The concentration of protein in the extracts was quantified using the Bio-Rad protein assay.

MTT Assay

Cell viability was determined using a MTT assay. After 24 hrs of culture in 96-well culture plates, cells were pre-treated with a number of protein kinase inhibitors in 100 μL of serum-free medium for 10 min before treatment with 100 μL of Pb^{2+} (100 μM) in serum-free medium. After 24 hrs, the medium was removed and 200 μL of MTT (0.5 mg/mL) in serum-free medium added to each well. After incubation at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 2 hrs, the medium was discarded, 200 μL of DMSO was added to each well, and the plate was incubated at 37°C for 5 minutes. The absorbance of the mixture was measured at 550 nm.

Immunoblotting

Aliquots of 30 μg of total protein were separated by electrophoresis on a Tris-Glycine gel (8-16% gradient) at 125 volts for 2 hrs. Proteins were then transferred to PVDF membranes, at 25 volts for 3 hrs. Membranes were blocked overnight with wash buffer (100 mM NaCl, 10 mM Tris.HCl, pH 7.4, and 0.1% Tween 20) containing 5% non-fat dry milk. Phosphorylated-nNOS was detected using polyclonal phospho-specific antibody (1 : 2,000), NP847, which identifies nNOS phosphorylated at Ser^{847}. When used in immunoblotting of proteins from CATH.a cells treated with Pb^{2+}, NP847 detects an immunoreactive band that corresponds to the 155-kDa nNOS. Following incubation with the primary antibody, membranes were washed five times with wash buffer, and the membrane incubated with anti-rabbit IgG-horseradish peroxidase conjugate (1 : 5,000) for 2 hrs at room temperature. Target bands were detected using the ECL™ Western blotting system (Amersham).

NADPH Oxidation

The rate of oxidation of NADPH by nNOS was determined spectrophotometrically. To remove L-arginine from the protein extract, aliquots were incubated with DOWEX 50W X4 resin in 40-mM Tris. HCl buffer, pH 7.4, at 4°C for 5 minutes. The mixture was then centrifuged at 13,000 rpm for 3 minutes and the supernatant added to a 80 mM Tris. HCl buffer containing 0.6 mM $CaCl_2$, 1 mM L-arginine, 4 μM FMN, 4 μM FAD and 4 μM BH4. The reaction was started by adding 0.2 mM of NADPH. The NO-mediated decrease of NADPH was monitored at 340 nm, at 25°C for 10 minutes, and the initial rate quantitated using the extinction coefficient, 6.22 $mM^{-1}\,cm^{-1}$.

Reductase Activities of nNOS

The reduction of cytochrome c and DCPI catalyzed by nNOS was determined spectrophotometrically in 0.2 mL of a 50 mM triethanolamine/ HCl buffer, pH 7.0, containing 0.1 mM NADPH, 4 μM FMN, and nNOS either in the presence (cytochrome *c*) or absence (DCPI) of Ca^{2+}/Calmodulin. The concentrations of the electron acceptors in the incubations were 100 μM Cytochrome c reduction was measured at 550 nm, at 25°C for 10 minutes, and initial rates calculated using the extinction coefficient for cytochrome c, 21 $mM^{-1}\,cm^{-1}$. DCPI reduction was measured at 600 nm, at 25°C for 1 minute, and initial rates calculated using the extinction coefficient for DCPI, 20.6 $mM^{-1}\,cm^{-1}$.

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