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Effects of Arginase Isoforms on NO Production by nNOS[1]

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Abstract

Both arginase isoforms (AI and AII) regulate high-level NO production by the inducible NOS, but whether the arginase isoforms also regulate low-level NO production by neuronal NOS (nNOS) is not known. In this study, 293 cells that stably overexpress nNOS gene (293nNOS cells) were transfected with rat AI (pEGFP-AI) or AII (pcDNA-AII) plasmids, and nitrite production was measured with or without supplemental L-arginine. Transfection with pEGFP-AI increased AI expression and activity 10-fold and decreased intracellular L-arginine by 50%. Nitrite production was inhibited by >80% when no L-arginine was supplemented but not when 1 mM L-arginine was present. The inhibition was reversed by an arginase inhibitor, N^{ω}-hydroxy-L-arginine. Transfection with pcDNA-AII increased AII expression and activity but had little effect on nitrite production even if no L-arginine was added. These results suggest that, in 293nNOS cells, AI was more effective in regulating NO production by nNOS, most likely by competing for L-arginine.

Keywords

transfection; N^{ω}-hydroxy-L-arginine; L-arginine; cell culture

Arginase is a hydrolase that metabolizes L-arginine to urea and L-ornithine. Two major isoforms of arginase have been identified: hepatic arginase (AI) and extrahepatic arginase (AII). AI is located in the cytosol and is most abundant in the liver, where its primary function is detoxification of ammonia to urea (1). AII is synthesized as an “immature” pre-protein which contains a putative presequence for mitochondrial import at its NH_2 terminus (2, 3). The presequence is cleaved to form “mature” AII after the “immature” form is imported into the mitochondria. AII is expressed in a number of extra-hepatic tissues, including blood vessels, small intestine, kidney, mammary gland, brain, and lungs (4-7).

Nitric oxide synthase (NOS) is a monooxygenase that converts L-arginine and molecular oxygen to NO and L-citrulline. Neuronal NOS (nNOS) is one of the three isoforms of NOS and is constitutively expressed in many vascular and neuronal tissues. nNOS produces low amounts of NO in the nanomolar range and is involved in the neurotransmission and regulation of vascular function (8, 9). This is in contrast to another isoform, inducible NOS

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(iNOS), which produces high levels of NO in the micromolar range and plays an important role in tissue inflammation and host defense.

The activity of arginase in cells may regulate NO biosynthesis via competition for the NOS substrate, L-arginine. Upregulation of either AI or AII appears to be equally effective in limiting NO production by iNOS (10-13), but the regulatory effect of arginase isoforms on NO production by nNOS has not been previously investigated. The effects seen in iNOS may not be applicable to nNOS because the rate of L-arginine utilization by nNOS is much lower. In addition, because AI and AII are located in different subcellular compartments, they may have different effects on L-arginine availability to nNOS. Defining the effects of arginase isoforms on nNOS is important pathogenetically and therapeutically because overproduction of NO by nNOS has been implicated in vascular, metabolic, and neurologic diseases.

The objective of this study was to compare the inhibitory effects of AI and AII on low output NO production by nNOS. We hypothesized that the cytosolic AI would be more effective than the mitochondrial AII in inhibiting NO production by the cytosolic nNOS. The hypothesis was tested by transfecting 293nNOS cells, a cell line stably expressing nNOS gene, with expression plasmids containing rat AI and AII genes and comparing NO production with or without supplemental L-arginine. We found that overexpression of AI was more effective in inhibiting NO production by nNOS than overexpression of AII was. The inhibitory effects were associated with lower intracellular L-arginine and were reversed if L-arginine was supplemented or if an arginase inhibitor was present.

METHODS

Construction of Expression Plasmids

The expression plasmid for rat arginase I, pEGFP-AI, was constructed by inserting the *Pst*I fragment of pARGr-2 (14) into the *Pst*I site of plasmid pEGFP-C3 (Clontech, La Jolla, CA), resulting in a fusion protein (GFP-AI) with GFP at the NH_2 terminus of AI. The expression plasmid for rat arginase II (pcDNA-AII) was constructed by inserting the *Pst*I fragment of pGEMrAII-2 (Accession No. U90887) (15) into the *Pst*I site of pcDNA-3C (Stratagene, La Jolla, CA). Correct orientation of the plasmids was documented with restriction digest analyses and DNA sequencing of the plasmids.

Cell Culture and Transfection

The 293nNOS cells are kidney embryonic cells stably transfected with nNOS and were a generous gift from Dr. Solomon Snyder (16). 293nNOS cells (passages 3–10) were seeded in duplicate in six well plates at a density of 1×10^6/well. Cells were grown in MEM (containing 0.6 mM L-arginine) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (P/S) and incubated at 37°C in a humidified atmosphere of 5% CO_2–21% O_2. At 70–80% confluency the cells were incubated for 3 h with 1 μg of pEGFP-AI, pcDNA-AII, or appropriate controls (pEGFP-C3 and pcDNA-3C) using the Lipofectamine Plus System (Gibco-BRL, Grand Island, NY) according to the protocol provided by the manufacturer. Cell medium was then removed and replaced with 1 ml of

10% MEM and 1% penicillin–streptomycin. Sixteen hours later, cells were gently rinsed with Kreb–Henseleit buffer (KHB, pH 7.4) and fresh KHB was added. The medium samples were collected for nitrite measurement by the Griess assay immediately, and cells were harvested for Western blot analysis and arginase activity measurement. Transfection with pEGFP-AI, pcDNA-AII, and control plasmids resulted in transient overexpression of these genes that was maximal at 16–24 h posttransfection.

Western Blot Analysis

293nNOS cells were lysed in 1% Triton lysis buffer containing 3% NP40, 50 mM EDTA, 150 mM NaCl, and antiproteases (0.4 mM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64), 2 mM 1.3-diisocoumarin, 2 mM 1,10-phenanthroline, 1 mM Na_3VO_4, and 1 mM NaF). The protein concentration was assessed by Bio-Rad assay (Bio-Rad, Hercules, CA). Twenty micrograms of each whole cell lysate was separated on 12% polyacrylamide gels and transferred to Millipore paper. The membranes were blocked with Tris-buffered saline with 0.05% Tween-20 and 5% milk overnight at 4°C. The blots were incubated with 1:3000 dilution of anti-AI polyclonal antibody, 1:3000 dilution of anti-AII polyclonal antibody (a gift from Dr. Christopher Jenkinson) for 1 h, and incubation with 1:2500 dilution of goat anti-rabbit horseradish–peroxidase labeled secondary antibody (Promega, Madison, WI) for 30 min. The signals were detected using a Renaissance chemiluminescence kit (NEN Life Science, Boston, MA) and were quantified by laser densitometry (LKB UltroscanXL).

Determination of Nitrite Production

Sixteen hours after transfection, cells were rinsed with KHB (pH 7.4) and the medium was replaced with fresh KHB containing either no L-arginine or 1 mM L-arginine. The concentration of nitrite in the medium was determined 2 h after fresh KHB was added using the Griess assay. The absorbance was read with a microplate reader (Thermomax, Molecular Devices, Sunnyvale, CA), and the areas were integrated with Softmax (version 2.32, Molecular Devices, Sunnyvale, CA). Standard curves were generated using known concentrations of nitrite in KHB and gave a linear response over the range 0.125–1 μM.

Determination of Arginase Activity

The arginase activity was determined in cell lysates by a modification of standard procedures for the detection of urea by reaction with diacetyl monoxime (5, 17). Cells were washed with KHB and harvested after 200 μL of arginase storage buffer (10 mM Tris, 10 mM glycine, 1 mM $MnCl_2$, and β-mercaptoethanol at pH 7.5) was added. The samples were then sonicated and centrifuged at 2000*g* at 4°C for 10 min. Ninety microliters of the supernatant was incubated for 5 min at 55°C with 10 ml of $MnCl_2$ (100 mM). Samples were then incubated for 1 h at 37°C with L-arginine (0.25 M). The reaction was stopped by adding 1 ml of diacetyl-monoxime in concentrated phosphoric acid–sulfuric acid. The samples were then vortexed, sealed, and heated in boiling water for 30 min. The urea content in each sample was then measured with a spectrophotometer at 490 nm. Freshly made solutions containing 0.1–0.9 μmol of urea were used to construct the standard curve. Arginase activity was expressed as nanomoles of urea per minute per milligram of protein.

Measurement of Amino Acids

293nNOS cells were transfected with 1 mg of pEGFP-AI, pcDNA-AII, and control plasmids as described previously. At the end of the 16-h incubation period, the cells were rinsed three times with cold KHB, scraped, and then extracted with lysis buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM sodium vanadate, 1 mM PMSF, 0.4 mM leupeptin, 1.6 mM pepstatin, and 1 mM NaF. The cell extracts were acidified with 10% salicylic acid to precipitate proteins. After centrifuge (10000g, 10 min, 4°C), 100 μL of supernatant was assayed for arginine, ornithine, and citrulline using an amino acid analyzer equipped with a lithium 10-cm ion-exchange column according to the manufacturer's protocols (Beckman 7300 high performance amino acid analyzer, Palo Alto, CA).

Experiments with N^{ω}-Hydroxy-L-arginine (HOA)

Sixteen hours after transfection with pEGFP-C3 and pEGFP-AI, cell medium was removed and replaced with KHB buffer containing 1 mM HOA (Sigma, St. Louis, MO) without L-arginine supplementation. Nitrite concentrations in cell media were measured.

Statistical Analysis

Data are presented as means ± standard errors (SE). To determine significant differences among various groups, one-way analysis of variance with Tukey's multiple comparison subtest was performed using commercially available software (Statview 512-T, Brainpower Company, Calabasas, CA). A P value of <0.05 was considered statistically significant.

RESULTS

Effect of Transfection on Expression of AI and AII

The nNOS293 cells constitutively expressed AII, which was inducible by dibutyric cAMP (data not shown). AI expression was not detectable by immunoblotting. Total arginase activity in the unstimulated 293nNOS cells was not affected by transfection with the two control plasmids (pEGFP-C3 and pcDNA-3C). The average arginase activity in unstimulated cells and cells transfected with two control plasmids was 38.6 ± 5.7 nmol urea/min/mg protein (n = 11).

Transfection of 293nNOS cells with pEGFP-AI significantly increased expression of GFP-AI protein (Fig. 1), and the arginase activity increased by 10-fold (557.9 ± 33.7 nmol urea/min/mg protein) (n = 6) compared to control cells (n = 11). With fluorescent microscopy, we estimated that the transfection efficiency was approximately 50% (Fig. 2).

Transfection with pcDNA-AII significantly increased expression of AII (Fig. 3), mostly as the mature form, and the arginase activity increased by 15-fold (1080.4 ± 76.4 nmol urea/min/mg protein) compared to pcDNA-3C (n = 6/group).

Effect of Overexpression of AI and AII on Intracellular Amino Acid Content

After 16 h of incubation in MEM that contains 0.6 mM L-arginine, the intracellular L-arginine content was 1.84 ± 0.21 nmol/mg protein (n = 4) in cells transfected with control

plasmids. In cells transfected with pEGFP-AI, the intracellular L-arginine was decreased to 1.09 ± 0.06 nmol/mg protein ($n = 4$, $P < 0.01$ vs control). In cells transfected with pcDNA-AII, the intracellular L-arginine content was 2.05 ± 0.09 nmol/mg protein ($n = 4$) (Fig. 4).

The intracellular L-ornithine content in pEGFP-AI-transfected cells (2.50 ± 0.03 nmol/mg protein, $P < 0.05$ vs other groups) was higher than that in cells transfected with the control plasmids (1.12 ± 0.06 nmol/mg protein) or pcDNA-AII (1.77 ± 0.05 nmol/mg protein) (Fig. 4). The intracellular L-citrulline content in pEGFP-AI transfected cells (0.41 ± 0.02 nmol/mg protein, $P < 0.001$ vs other groups) was lower than that in cells transfected with the control plasmids (0.90 ± 0.07 nmol/mg protein) or pcDNA-AII (0.99 ± 0.05 nmol/mg protein).

Effects of Exogenous L-Arginine on Nitrite Production in nNOS293 Cells

Because the intracellular L-arginine concentration was decreased in AI-transfected cells, we sought to determine whether nitrite production was limited and depended on exogenous L-arginine. The cells were rinsed with KHB at the end of 16 h of incubation. The cells were then exposed to 0 or 1 mM L-arginine in KHB for 2 h.

Over 2 h, 293nNOS cells transfected with pEGFP-C3 produced 0.35 ± 0.07 $\mu M/10^6$ cells ($n = 6$) of nitrite at 1 mM L-arginine. Decreasing L-arginine in the medium to 0 mM did not affect nitrite production (0.29 ± 0.04 $\mu M/10^6$ cells, $n = 9$) (Fig. 5). Overexpression of GFP-AI did not affect nitrite production when the medium contained 1.0 mM L-arginine (0.31 ± 0.06 $\mu M/10^6$ cells, $n = 6$), but it inhibited nitrite production by greater than 80% when exogenous L-arginine was absent (Fig. 5).

293nNOS cells transfected with pcDNA-3C produced 0.29 ± 0.14 $\mu M/10^6$ cells ($n = 7$) of nitrite at 1 mM L-arginine and 0.22 ± 0.02 $\mu M/10^6$ cells at 0 mM L-arginine. Despite the dramatic increase in arginase activity, cells transfected with pcDNA-AII did not decrease nitrite production at 0 mM exogenous L-arginine. The nitrite production was 0.28 ± 0.07 $\mu M/10^6$ cells for pcDNA-AII-transfected cells ($n = 7$) at 1 mM L-arginine and 0.23 ± 0.01 $\mu M/10^6$ cells at 0 mM L-arginine (Fig. 5).

Effect of N^{ω}-Hydroxy-L-Arginine

N^{ω}-Hydroxy-L-arginine, an intermediate in the NOS-catalyzed conversion of L-arginine to NO, is a potent inhibitor of arginase (18-20). To determine if the inhibition of NO production in pEGFP-AI-transfected cells in the absence of supplemental L-arginine was due to decreases in L-arginine available to nNOS, nitrite production was assessed in cells incubated with 1 mM HOA for 2 h without added L-arginine ($n = 5$). HOA restored nitrite production to 0.37 ± 0.13 $\mu M/10^6$ cells in pEGFP-AI-transfected cells, similar to that seen with 1 mM L-arginine without HOA treatment (0.31 ± 0.06 $\mu M/10^6$ cells) ($n = 6$) (Fig. 6).

DISCUSSION

The objective of our study was to determine how arginase isoforms regulate NO production by nNOS. To investigate the specific effects of AI and AII, we constructed plasmids carrying either AI or AII genes and transfected 293nNOS cells with these plasmids. After 16 h

of incubation in medium containing 0.6 mM L-arginine, 293nNOS cells transfected with pEGFP-AI had a 50% lower intracellular arginine content than pcDNA-AII-transfected cells or control cells. Although the steady-state concentrations of amino acid reflect only the balance between production and utilization of the amino acid, these data were consistent with higher arginine utilization by arginase I pathways.

Overexpression of AI inhibited nitrite production by greater than 80% if no exogenous L-arginine was supplemented following 16 h of incubation. The inhibition could be reversed if 1 mM L-arginine was added to the medium or if 1 mM HOA, an arginase inhibitor, was added. HOA can be metabolized to NO by NOS or oxidants in some cell types (21) but not in others (10, 22). In nNOS293 cells treated with 1 mM HOA, nitrite production did not increase. Therefore, the main effect of HOA seems to be arginase inhibition, but we cannot exclude the possibility that small amounts of NO may derive from HOA directly. Taken together, these results indicate that, when the extracellular L-arginine was 0.6 mM, a 10-to 15-fold increase in AI activity was sufficient to decrease the intracellular L-arginine to a level that limits NO production by nNOS.

In contrast, transfection with pcDNA-AII, which significantly increased the expression of mature native AII, did not inhibit nitrite production even if no L-arginine was supplemented after a 16-h incubation in medium containing 0.6 mM L-arginine. The intracellular L-arginine and L-citrulline concentrations were unchanged, but L-ornithine increased by approximately 60%. We also noted that nNOS293 cells continue to produce NO in the absence of exogenous L-arginine despite their basal AII activity being severalfold higher than that of nonhepatic cells. These results indicate that in nNOS293 cells AII does not seem to regulate NO production by nNOS. These results also raise the question about the kinetic relationship between mitochondrial L-arginine and cytosolic L-arginine. It has recently been shown that mitochondria from rat brain seem to possess high-affinity arginine transporters kinetically similar to the y+ systems on plasma membrane (23). If so, the mitochondrial arginine uptake could be regulated independent of the concentration of L-arginine in the cytoplasm, and arginine concentration in the mitochondria could be affected by cationic amino acids (e.g., ornithine, lysine) in the cytoplasm that also compete for the same transport pathway.

Previous studies investigating interactions between arginase and NOS pathways have focused mostly on high-output NO produced by iNOS (24, 25). In rat aortic endothelial cells, induction of AI limited NO production by iNOS induced by lipopolysaccharide (LPS) (10). In J744A.1 mouse macrophages, inhibition of arginase activity increased NO production by iNOS induced by LPS, and the effect was most pronounced at low exogenous L-arginine (11). In unstimulated and stimulated rodent alveolar macrophages that expressed iNOS, inhibition of arginase caused a shift of L-arginine metabolism to the iNOS pathway, and the extent of the shift was most complete when a high level of iNOS was induced by LPS (12). In RAW246.7 cells, increased iNOS activity by LPS was further enhanced by γIFN, which simultaneously inhibited arginase activity (13, 15, 24). Competition for L-arginine between arginase and iNOS pathways was also implicated in macrophages treated with TH1 vs TH2 cytokines (26, 27). None of these studies, however, compared the relative potency of AI and AII in regulating NO production, but it seemsthat, when NO production

is high (in micromolar range), both arginase isoforms are equally effective in inhibiting NO production by limiting L-arginine availability to iNOS.

Our study was different from these previous studies in two important aspects. First, we focused on the effects of arginase on NO produced by a constitutive NOS. 293nNOS cells produced NO at a rate which was several orders of magnitude lower than that of cells expressing iNOS. Second, our study compared the efficacy of the two known arginase isoforms on NO production using gene-transfer technology instead of cytokines. We showed that nitrite production by nNOS was inhibited when the cytosolic AI activity was increased but not when the mitochondrial AII activity was increased. In a recent study, overexpression of trangene AII also inhibited NO production by eNOS in human umbilical vein endothelial cells (HUVEC), although the effect was smaller than that of transgene AI (28). The discrepancy may be related to several factors, including different subcellular localization of nNOS vs eNOS, mechanisms for arginine transport on mitochondrial membrane, and the status of mitochondrial NOS in these two cell types. Mitochondrial NOS has been shown to be present in the cells of brain, liver, and skeletal muscle as well as some transformed cells (PC-12 and COS-1) (29, 30). It is unclear whether or not the mitochondrial NOS activity is different between HUVEC cells and nNOS 293 cells.

In summary, we showed that AI was more effective in regulating NO production by nNOS, mostly likely by competing for L-arginine. Since overproduction of NO by nNOS has been implicated in the pathogenesis of many vascular, metabolic, and neurological diseases, gene therapy with AI may be a unique tool for modulating intracellular NO production in these nNOS-related diseases.

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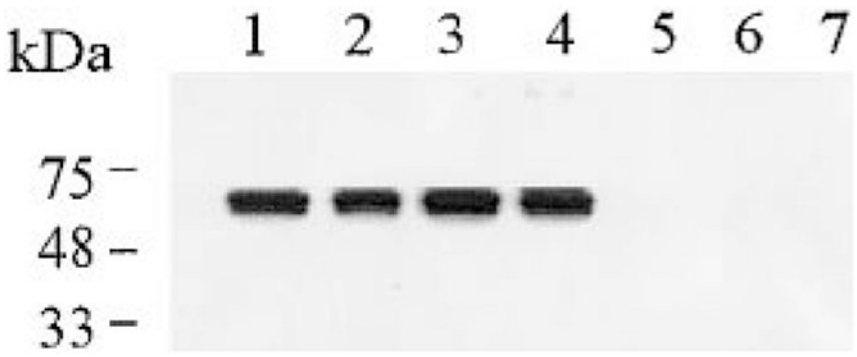

FIG. 1.

Western blot analysis of AI from cells transfected with pEGFP-AI expression plasmid: lanes 1–4, pEGFP-AI; lanes 5–7, pEGFP-C3. Each lane represents different experiments. Protein concentration for each lane: 20 μg.

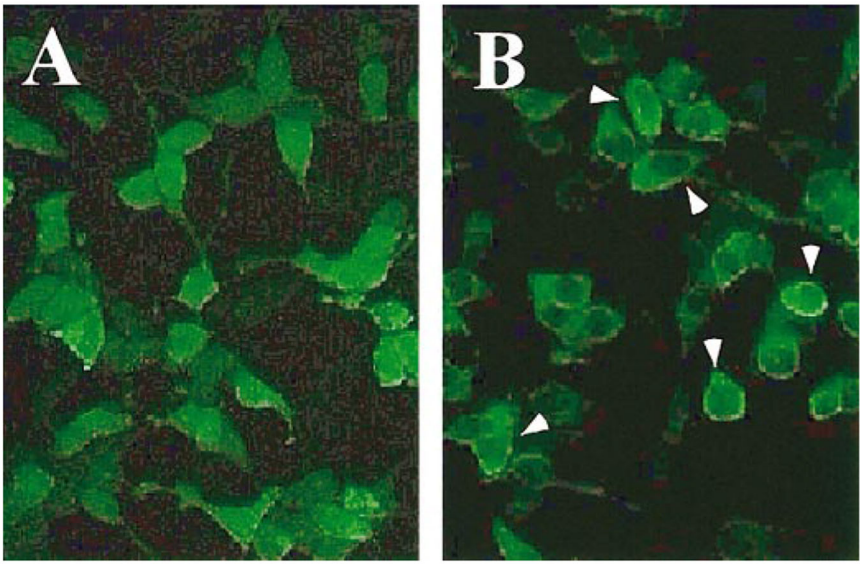

FIG. 2.

Fluorescent microscopic pictures of 293nNOS cells transfected with pEGFP-C3 (A) and pEGFP-AI (B). Arrowheads indicate cells expressing GFP-AI. Magnification: 60×.

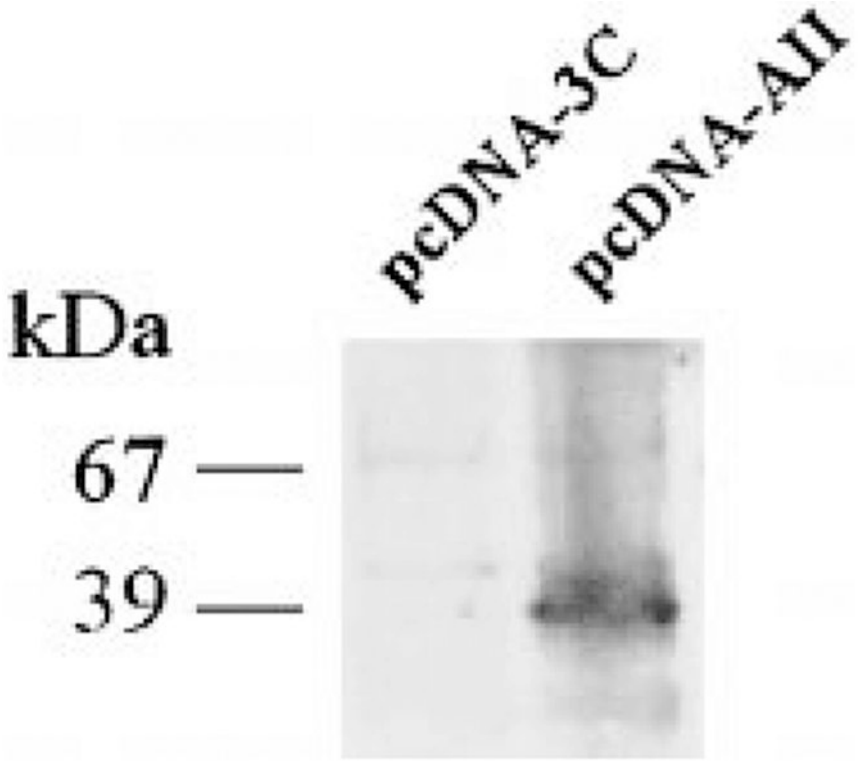

FIG. 3.

Western blot analysis of AII from cells transfected with pcDNA-AII expression plasmid: lane 1, pcDNA-3C; lane 2, pcDNA-AII. Protein concentration for each lane: 20 μg.

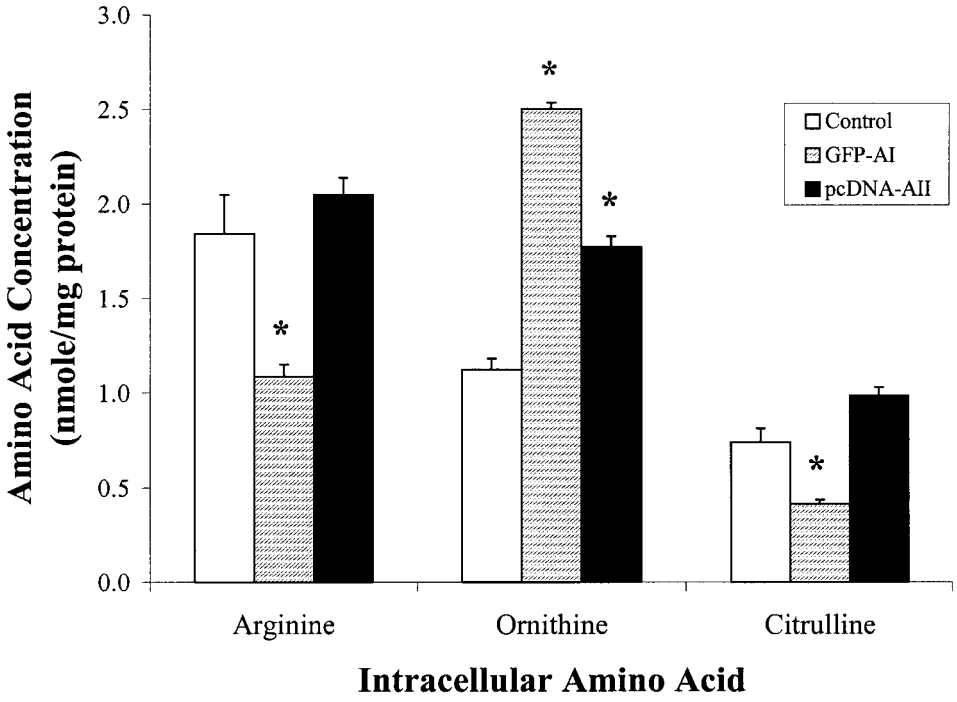

FIG. 4.

Intracellular arginine, citrulline, and ornithine in cells 16 h after incubation with control, pEGFP-AI, and pcDNA-AII plasmids. *$P < 0.05$ vs control plasmids.

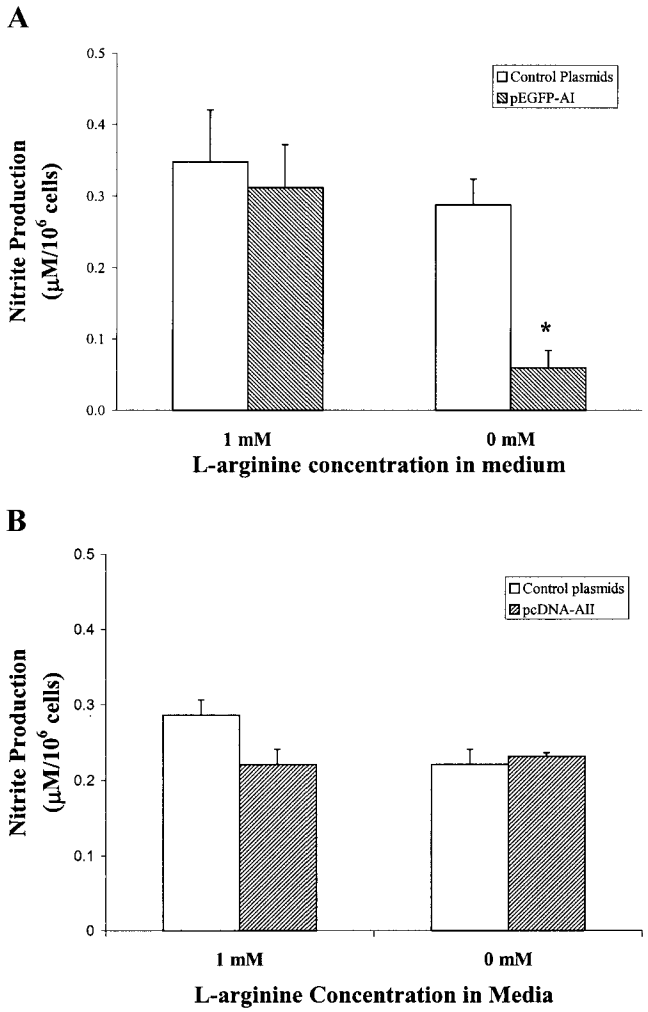

FIG. 5.

Nitrite production by 293nNOS cells incubated with (A) pEGFP-AI ($n = 6$) and (B) pcDNA-AII ($n = 6$) for 16 h and exposed to 0 or 1 mM L-arginine in the medium for 2 h. *$P < 0.05$ vs pEGFP-C3.

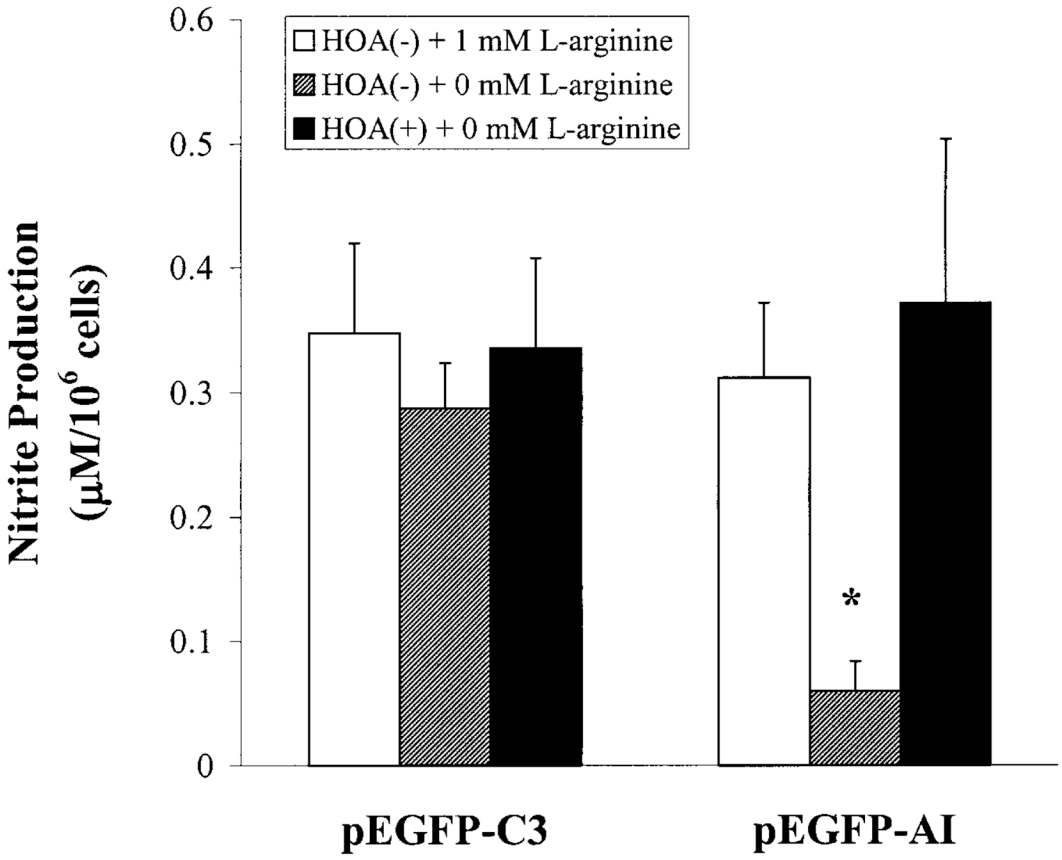

FIG. 6.

Nitrite production by 293nNOS cells incubated with control, pEGFP-AI, and pcDNA-AII plasmids for 16 h and exposed to 0 mM L-arginine in the medium for 2 h. Some cells were treated with 1 mM N^{ω}-hydroxy-L-arginine added at the beginning of the 2-h incubation. Nitrite production by 293 cells incubated with control, pEGFP-AI, and pcDNA-AII plasmids for 16 h and exposed to 1 mM L-arginine in the medium for 2 h is also shown for comparison. *$P < 0.05$ vs 0 mM L-arginine without N^{ω}-hydroxy-L-arginine; $N = 5$ each for experiments with N^{ω}-hydroxy-L-arginine.