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Pharmacological Interference with Dimerization of Human Neuronal Nitric-Oxide Synthase Expressed in Adenovirus-Infected DLD-1 Cells

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ABSTRACT

A recombinant adenovirus containing the cDNA of human neuronal nitric-oxide synthase (nNOS) was constructed to characterize the interaction of nNOS with *N*-[(1,3-benzodioxol-5-yl)methyl]-1-[2-(1H-imidazole-1-yl)pyrimidin-4-yl]-4-(methoxycarbonyl)-piperazine-2-acetamide (BBS-1), a potent inhibitor of inducible NOS dimerization [*Proc Natl Acad Sci USA* **97:**1506–1511, 2000]. BBS-1 inhibited de novo expression of nNOS activity in virus-infected cells at a half-maximal concentration (IC_{50}) of 40 ± 10 nM in a reversible manner. Low-temperature gel electrophoresis showed that BBS-1 attenuated the formation of SDS-resistant nNOS dimers with an IC_{50} of 22 ± 5.2 nM. Enzyme inhibition progressively decreased with increasing time of addition after infection. BBS-1 did not significantly inhibit dimeric nNOS activity ($IC_{50} > 1$ mM). Long-term incubation with BBS-1 of human embryonic kidney cells stably transfected with nNOS or endothelial NOS revealed a slow time- and concentration-dependent decrease of NOS activity with half-lives of 30 and 43 h and IC_{50} values of 210 ± 30 nM and 12 ± 0.5 μM, respectively. These results establish that BBS-1 interferes with the assembly of active nNOS dimers during protein expression. Slow inactivation of constitutively expressed NOS in intact cells may reflect protein degradation and interference of BBS-1 with the de novo synthesis of functionally active NOS dimers. As time-dependent inhibitors of NOS dimerization, BBS-1 and related compounds provide a promising strategy to develop a new class of selective and clinically useful NOS inhibitors.

The biological messenger nitric oxide (NO) plays an important role in the regulation of various biological processes such as vasodilation, neurotransmission, and host-defense against pathogens (Mayer and Hemmens, 1997), but overproduction of NO may have deleterious effects in infectious and inflammatory diseases (Moncada and Higgs, 1995; Colasanti and Suzuki, 2000). Cytokine-induced expression of inducible nitric-oxide synthase in vascular smooth muscle under conditions of severe sepsis may contribute to lethality through increased vascular leakage and life-threatening hypotension. In addition, excess NO from iNOS may cause tissue injury in long-term diseases, in particular rheumatoid arthritis (Amin et al., 1999) and neurodegenerative disorders such as stroke and Parkinson's disease (Heneka and Feinstein, 2001). Therefore, isoform-selective NOS inhibitors are required that can limit harmful NO overproduction caused by iNOS or neuronal NOS (nNOS) without affecting endothelial NO synthesis which is essential for vascular homeostasis (Feron, 1999).

Enzymatic formation of NO from L-arginine is catalyzed by three NOS isoforms [EC 1.14.13.39; reviewed in Pfeiffer et al. (1999)]. The nNOS and endothelial NOS (eNOS) isoforms are constitutively expressed and activated by hormones and neurotransmitters that increase the intracellular concentration of free Ca^{2+}, whereas a Ca^{2+}-independent isoform (iNOS) becomes induced in most types of mammalian cells in response to inflammatory cytokines. L-Arginine is oxidized by the N-terminal oxygenase domain, which contains a P450-type heme and binds the pterin cofactor BH_4. The NADPH-

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ABBREVIATIONS: NO, nitric oxide; NOS, nitric-oxide synthase; nNOS neuronal NOS (type I); iNOS, inducible NOS (type II); eNOS, endothelial NOS (type III); BH_4, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; DAHP, 2,4-diamino-6-hydroxy-pyrimidine; BBS-1, *N*-[(1,3-benzodioxol-5-yl)methyl]-1-[2-(1H-imidazole-1-yl)pyrimidin-4-yl]-4-(methoxycarbonyl)-piperazine-2-acetamide; DMEM, Dulbecco's modified Eagle's medium; CMV, cytomegalovirus; FCS, fetal calf serum; GFP, green fluorescence protein; HEK, human embryonic kidney; hnNOS-Ad, Adenovirus encoding human neuronal nitric oxide synthase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; LT, low temperature; PAGE, polyacrylamide gel electrophoresis; L-NNA, N^G-nitro-L-arginine.

derived electrons required for reductive O_2 activation are transferred via the flavin-containing C-terminal reductase domain to the catalytic heme site. This process is under the control of Ca^{2+}/calmodulin binding. All active NOS isoforms are homodimers. Electron transfer in NOS requires monomer dimerization, because it occurs "in trans" from the reductase domain of one subunit to the heme in the oxygenase domain of the other subunit (Siddhanta et al., 1998). This feature of NOS has important consequences because it predicts that NOS monomers, even if they contain tightly bound heme, will not catalyze O_2 reduction, a reaction that results in formation of superoxide and H_2O_2 in the absence of bound L-arginine or BH_4 (Gorren and Mayer, 1998).

Virtually all potent NOS inhibitors described so far are structural analogs of L-arginine or BH_4, which competitively antagonize substrate or pterin binding. However, clinical use of these drugs has been limited because of their relatively low potency, insufficient iNOS selectivity, or in vivo toxicity in laboratory animals (Mayer and Andrew, 1998). Considering the essential role of homodimerization for the assembly of active NOS, dimerization inhibitors might prove useful to interfere with the expression of active iNOS while leaving the constitutively expressed isoforms unaffected. The first class of compounds reported to weakly inhibit iNOS dimerization were antifungal imidazoles (Sennequier et al., 1999). Using combinatorial chemical libraries, McMillan et al. (2000) independently discovered that pyrimidineimidazoles are potent iNOS-selective dimerization inhibitors. The compound *N*-[(1,3-benzodioxol-5-yl)methyl]-1-[2-(1H-imidazole-1-yl)pyrimidin-4-yl]-4-(methoxycarbonyl)-piperazine-2-acetamide (BBS-1) exhibited high affinity for iNOS (K_d = 2.2 nM) with about 5,000- and 1,000-fold selectivity over nNOS and eNOS dimerization, respectively. Using gel filtration chromatography, X-ray crystallography, and biochemical techniques, BBS-1 was shown unequivocally to inhibit iNOS by interfering with protein dimerization.

Although BBS-1 is a very weak inhibitor of eNOS dimerization, the selectivity of the drug toward nNOS is relatively modest. Moreover, it is conceivable that nNOS and eNOS may become sensitive to dimerization inhibitors because of interference of these drugs with de novo synthesis of the active proteins upon long-term exposure. In the present study, we investigated the effects of BBS-1 on the expression of nNOS activity in DLD-1 cells infected with an adenovirus encoding the human nNOS gene (He et al., 1998) and studied the long-term effects of BBS-1 on constitutively expressed NOS activity in HEK 293 cells stably transfected with human nNOS and eNOS (Schmidt et al., 2001).

Materials and Methods

Materials. L-[2,3,4,5-^{3}H]Arginine hydrochloride (57 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and purified by high performance liquid chromatography as described previously (Klatt et al., 1996b). BH_4 was from B. Schircks Laboratories (Läufelfingen, Switzerland). Recombinant rat nNOS was purified from baculovirus-infected Sf9 cells as described previously (Harteneck et al., 1994; Mayer et al., 1996a). BBS-1 was synthesized by Dr. Gary Phillips and David Davey (Berlex Biosciences, Richmond, CA) and provided as a 100 mM stock solution in dimethyl sulfoxide. DLD-1, COS-7, HEK 293, and porcine aortic endothelial cells were cultured in DMEM (Sigma), containing 100 U/ml penicillin, 100 U/ml streptomycin, 1.25 μg/ml amphotericin, and 10% fetal calf serum (PAA Laboratories GmbH, Pasching, Austria). NOS-transfected HEK 293 cells were grown in the presence of 250 μg/ml G-418 (Schmidt et al., 2001). Endothelial cells were isolated as described previously (Schmidt et al., 1989). Materials for molecular biology were from New England Biolabs (Beverly, MA), Invitrogen (Carlsbad, CA), and QIAGEN (Valencia, CA). All other chemicals were from Sigma (St. Louis, MO).

Preparation of Plasmid DNA. The baculovirus vector pVL1393, containing the human nNOS cDNA, was cut with *Eco*RI (5′ and 3′ termini), followed by refilling the 3′ termini with Klenow fragment of *Escherichia coli* DNA polymerase I and digestion of the 5′ termini with *Xba*I. After cutting the shuttle vector pAdTrack-CMV, containing a GFP sequence, a cytomegalovirus promoter region, and a polyadenylation site (He et al., 1998) with *Eco*RI and *Xba*I, the two plasmids were ligated using the Rapid DNA Ligation Kit (Roche Diagnostics, Indianapolis, IN). After a control digestion with *Pme*I, the ligation product was amplified by transformation into *E. coli* Top 10 cells in Luria broth medium containing 50 μg/ml kanamycin.

Homologous Recombination. Active virus was generated as described previously (He et al., 1998). Briefly, the pAdEasy-1 vector and the human nNOS containing shuttle vector (hnNOS-pAdTrack-CMV; linearized with *Pme*I), were cotransformed into BJ5183 cells by electroporation. Colonies were selected on Luria broth medium/kanamycin plates and grown to prepare the recombinant plasmids. DNA isolated from a positive clone, selected after control digestion with *Bam*HI, and amplified by transformation into *E. coli* Top 10, was digested with *Pac*I and used for transfection into HEK 293 cells. The cells were grown to about 80% confluence on 75-cm^2 flasks, washed with FCS-free DMEM, incubated for 15 min in FCS-free DMEM, followed by addition of 30 μg of the *Pac*I-digested DNA [in 750 μl of FCS-free DMEM plus 60 μl of LipofectAMINE (Invitrogen)]. Nondigested DNA was used as control. Five hours later, the mixture was replaced by 12 ml of DMEM containing 10% FCS. After 18 h, the cells were spread out onto three 75-cm^2 flasks and grown until several plaques had appeared in the cell monolayers (usually after 7–14 days) with change of medium every 2 days. Cells were harvested by centrifugation for 5 min at 2000*g* at 4°C. The pellet was resuspended in 10 ml of FCS-free DMEM and lysed by four cycles of freezing in ethanol/dry ice and rapid thawing at 37°C. After centrifugation for 10 min at 2000*g*, the supernatants were pooled (approximate total volume, 65 ml). Before further amplification of the virus, nNOS expression was verified by Western blotting in virus-infected COS-7 cells (see below).

Large Scale Amplification and Purification of Adenovirus. HEK 293 cells were grown in 14 175-cm^2 flasks to 90% confluence. The medium was removed followed by the addition of 20 ml of FCS-free DMEM and 4.5 ml of virus-containing supernatant to each flask. Two hours later, 20 ml of DMEM with FCS (10% final) was added. Cells were checked for expression of the viral genome by monitoring GFP expression and harvested after 40 to 70 h by centrifugation at 1,300*g* for 5 min. The pellet was resuspended in 20 ml of FCS-free DMEM and lysed by four cycles of freeze/thawing. Five milliliters of the lysate were loaded onto a discontinuous CsCl gradient (1.40 and 1.25 g/ml, 2.5 ml each) in 17 ml of polyallomer tubes (Kendro) and centrifuged for 5 h at 52,000*g*. The white adenovirus bands were then extracted from the tubes (0.5–1 ml) with needle and syringe, injected into a Slide-A-Lyzer Dialysis Cassette (Pierce, Rockford, IL), and dialyzed twice (5 h and overnight) at 4°C against 2 liters of Tris-HCl buffer, pH 7.5 (25 mM Tris-HCl, 137 mM NaCl, 6 mM KCl, and 0.7 mM Na_2HPO_4). The virus stock solution was stored at −70°C in 10% glycerol. For further amplification, an appropriate amount of the virus stock was used to infect HEK 293 cells (10–16 175-cm^2 flasks), followed by purification of the adenovirus as described above.

Viral Infection of COS-7 and DLD-1 Cells. Cells were grown on six-well plates to about 90% confluence in DMEM with 10% FCS. Before infection, cells were washed with potassium phosphate buffer, pH 7.4, and incubated with 0.5 ml of FCS-free DMEM and 5 to 50 μl

of viral stock solution. Two hours later, DMEM, 10% FCS, and 7.4 μM hemin chloride were added, and infection was monitored as GFP expression. NOS expression was verified by immunoblotting of cell lysates. For this purpose, the 1,300*g* cell pellet was resuspended in triethanolamine/HCl buffer, pH 7.4, containing 14 mM 2-mercaptoethanol, and 0.5 mM EDTA, followed by three cycles of rapid freeze/thawing in liquid nitrogen. NOS activity was determined in both intact cells and homogenates as described below.

Purification of Human nNOS from Adenovirus-Infected DLD-1 Cells. Human nNOS was purified from DLD-1 cells as described for rat nNOS purification from baculovirus-infected Sf9 cells (Mayer et al., 1996) with slight modifications to account for the relatively low amounts of available protein. Cells from 10 Petri dishes (diameter 90 mm) cultured with or without 10 μM BBS-1 were harvested and lysed 40 h after infection by freeze/thawing. Cell lysates were centrifuged for 15 min at 18,000*g* at 4°C, and the supernatants were loaded onto 2′,5′-ADP-Sepharose columns with a bed volume of ~0.3 ml. The columns were washed twice as described previously (Mayer et al., 1996), and bound protein was eluted with triethanolamine/HCl, pH 7.4, containing 10 mM NADPH. The eluates (~0.5 ml) were stored at −70°C. Protein was determined using bovine serum albumin as standard (Bradford, 1976).

Determination of NOS Activity in Intact Cells. Intact cells were washed and incubated at 37°C with 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2.5 mM $CaCl_2$. Reactions were started by addition of 0.3 μM Ca^{2+}-ionophore A23,187 and [^{3}H]arginine (~500,000 dpm). After 10 min, cells were washed twice with ice-cold 50 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 0.1 mM EDTA and lysed by incubation with 1 ml of 0.01 M HCl for 90 to 120 min. Aliquots (0.1 ml) were removed for measurement of incorporated radioactivity. To the remaining samples (0.9 ml), 0.05 to 0.1 ml of 0.2 M sodium acetate buffer, pH 13.0, containing 10 mM L-citrulline was added (final pH, 5.1). [^{3}H]Citrulline was separated from [^{3}H]arginine by cation-exchange chromatography as described previously (Schmidt and Mayer, 1999). Values are expressed as percentage conversion of incorporated [^{3}H]arginine.

Determination of NOS Activity in Cell-Free Preparations. NOS activity was measured with the citrulline assay as described previously (Schmidt and Mayer, 1999). Protein fractions (purified enzymes or cell lysates) were incubated for 10 min at 37°C in 0.1 ml of 50 mM triethanolamine/HCl buffer, pH 7.4. Unless indicated otherwise, reaction mixtures contained 0.1 mM [^{3}H]arginine (~60,000 cpm), 0.5 mM $CaCl_2$, 10 μg/ml calmodulin, 0.2 mM NADPH, 10 μM BH_4, 5 μM FAD, 5 μM FMN, 0.2 mM CHAPS, and 12 mM β-mercaptoethanol. Blank values were determined in the absence of enzyme. Uncoupled activity of nNOS was measured as calmodulin-dependent formation of H_2O_2 as described previously (Heinzel et al., 1992).

Determination of Lactate Dehydrogenase Release from DLD-1 Cells. DLD-1 cell supernatant (0.1 ml) was mixed with 0.2 ml of a reagent containing 10 mM pyruvate and 1.0 mM NADH in 44 mM potassium phosphate buffer, pH 7.5. The decrease of absorption at 340 nm (ϵ = 6.22 mM/cm) was monitored. Total lactate dehydrogenase content was determined by treating the cells with 1% Triton X-100.

LT-PAGE and Western Blotting. NOS dimerization was analyzed by LT-PAGE as described previously (Klatt et al., 1995). Briefly, cell lysates (5–10 μg of total protein) were incubated for 10 min at 37°C in 50 mM triethanolamine-HCl buffer, pH 7.4 , containing 0.2 mM BH_4 in a total volume of 24 μl. Samples were put on ice, and 6 μl of 5-fold Laemmli buffer (Laemmli, 1970), including 25% (v/v) 2-mercaptoethanol, 10% SDS, 50% glycerol, and 0.02% bromphenol blue was added, followed by gel electrophoresis on 5% polyacrylamide gels, using the Mini Protean II system from Bio-Rad (Vienna, Austria). Gels and buffers were equilibrated at 4°C, and the buffer tank was cooled in an ice bath. The separated proteins were transferred to nitrocellulose membranes by electroblotting at 240 mA for 90 min, followed by detection with anti-nNOS antibodies (1:5000 dilution) as described previously (Golser et al., 2000). Monomer/dimer ratios were quantified with a Hoefer Double Vision UV/VIS lamp/video camera and the software supplied by the manufacturer.

Determination of BH_4 Levels in DLD-1 Cells. DLD-1 cells were incubated for 48 h with or without 10 mM DAHP on four petri dishes (diameter, 90 mm), followed by the determination of BH_4 levels by high-performance liquid chromatography as described previously (Klatt et al., 1996a).

Results

Characterization of the Adenovirus nNOS Overexpression System. Infection of DLD-1 cells with increasing amounts of purified adenovirus encoding human nNOS resulted in expression of NOS activity 24 h after infection, as shown by increased conversion of [^{3}H]arginine to [^{3}H]citrulline by the intact cells stimulated with 0.3 μM Ca^{2+}-ionophore. Upon addition of 30 to 50 μl of the virus solution, about 80% of the incorporated radioactive substrate had been converted to L-citrulline. Half-maximal effects were observed with about 6 μl of virus solution (Fig. 1A). Further experiments were performed with 15 to 20 μl of the virus stock solution. Under these conditions, approximately 90% of the cells expressed GFP (data not shown), and [^{3}H]arginine-to-[^{3}H]citrulline conversion was 50 to 60%. Adenovirus infection did not cause detectable release of lactate dehydrogenase activity compared with controls (~8% of total amount releasable by Triton X-100; data not shown). As expected, the nonselective NOS inhibitor L-NNA led to a concentration-dependent decrease of L-arginine conversion with an IC_{50} of 0.54 ± 0.07 μM (Fig. 1B). About 6 to 10% of the incorporated [^{3}H]arginine was converted to [^{3}H]citrulline (or a product with citrulline-like chromatographic behavior) even in the presence of 1 mM L-NNA. As shown in Fig. 1C, L-NNA–insensitive L-arginine conversion was virtually identical to that seen with noninfected cells or cells treated with 10 μM BBS-1 2 hours after infection. As shown previously for NOS overexpression in other cell types (Albakri and Stuehr, 1996; Klatt et al., 1996b), NOS activity of adenovirus-infected DLD-1 cells was slightly increased when hemin chloride (7.4 μM) was present during the infection period (Fig. 1C). Hemin chloride was thus always added to increase heme availability and facilitate NOS dimerization. Uptake of [^{3}H]arginine (10–15% of added radioactivity) was not significantly affected by nNOS overexpression, addition of hemin chloride, or enzyme inhibitors (Fig. 1D).

To test for Ca^{2+} sensitivity of overexpressed nNOS, enzyme activity was measured in DLD-1 cells treated with and without Ca^{2+} ionophore A23,187 (0.3 μM) in the absence and presence of extracellular Ca^{2+} ions (Fig. 1E). Under nominally Ca^{2+}-free conditions (0.1 mM EGTA), 14 ± 0.7% of the incorporated [^{3}H]arginine was converted to [^{3}H]citrulline. This activity was slightly but significantly higher than basal (see Fig. 1C above), indicating that the enzyme was partially active in resting cells. L-Arginine conversion was about 25% in the presence of 2.5 mM extracellular $CaCl_2$ and further increased to 45 ± 4.3% when the Ca^{2+} ionophore was additionally present.

Specific NOS activity of DLD-1 cell lysates was 0.72 ± 0.06 and 1.5 ± 0.11 nmol/min/mg in the absence and presence of

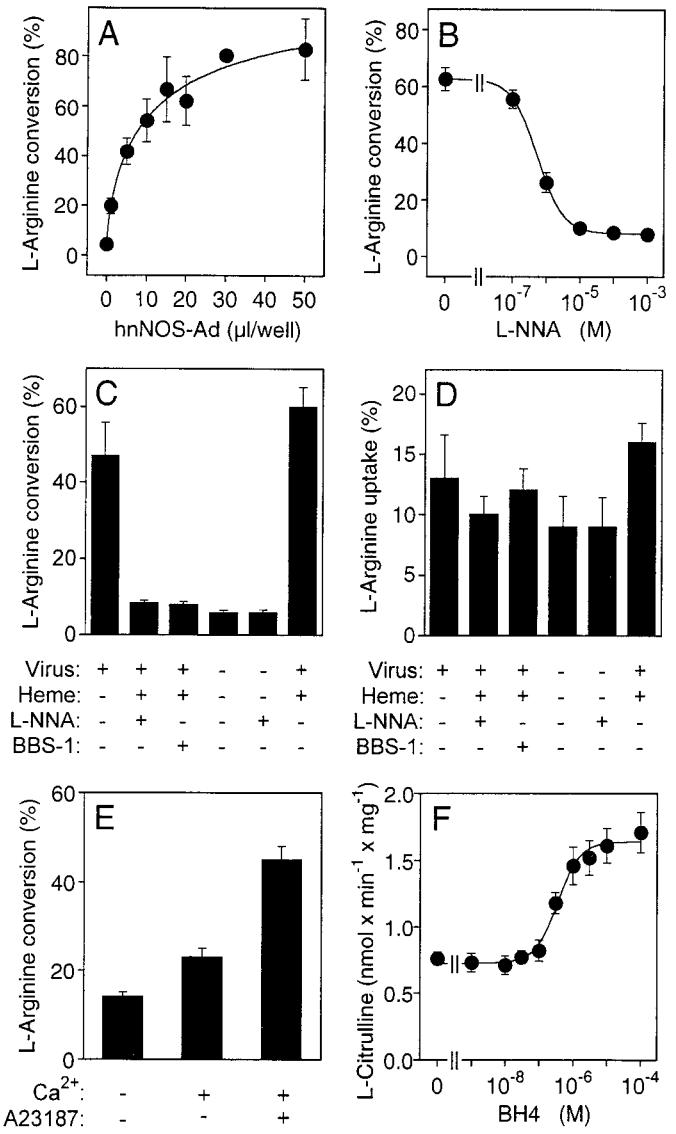

Fig. 1. Expression of human nNOS in adenovirus-infected DLD-1 cells. A, effect of increasing amounts of hnNOS-Ad solution. NOS activity was determined 24 h after infection as conversion of [^{3}H]arginine to [^{3}H]citrulline by the intact cells stimulated with Ca^{2+} ionophore A23,187 (0.3 μM). Results are mean values ± S.E.M. of four duplicate determinations performed with two different virus preparations (two assays with each preparation). B, concentration-dependent inhibition of nNOS by L-NNA. DLD-1 cells were infected with hnNOS-Ad for 24 h, followed by incubation for 30 min with the indicated concentrations of L-NNA, and determination of NOS activity in A23,187-stimulated cells. C, effects of hemin chloride (heme; 7.4 μM), L-NNA (0.1 mM), and BBS-1 (10 μM) on expression of NOS activity. L-NNA was added 30 min before activity measurements, and hemin chloride and BBS-1 were added 2 h after the virus; control cells received vehicle instead of virus solution. NOS activity was determined in A23,187-stimulated cells 24 h after infection. D, effects of hemin chloride, L-NNA, and BBS-1 on [^{3}H]arginine uptake. Experimental conditions as in C. Uptake was measured for 10 min and is expressed as percentage of added radioactivity (~500,000 dpm). E, effects of extracellular Ca^{2+} and Ca^{2+} ionophore A23,187 on NOS activity in DLD-1 cells infected with hnNOS-Ad (15 μl, 24 h). Nominally Ca^{2+}-free conditions were achieved by incubating the cells in the presence of 0.1 mM EGTA. Where indicated, $CaCl_2$ (2.5 mM final) and A23,187 (0.3 μM final) were added before determination of [^{3}H]arginine-to-[^{3}H]citrulline conversion. F, BH_4-dependent NOS activity in DLD-1 cell lysates 24 h after infection with hnNOS-Ad (15 μl). Enzyme activity was determined with the citrulline assay under standard conditions (see *Materials and Methods*) in the presence of the indicated concentrations of BH_4. B–F, data are mean values ± S.E.M. of three experiments.

10 μM BH_4, respectively. The EC_{50} of BH_4 for enhancing NOS activity was 0.43 ± 0.06 μM (Fig. 1F).

Effects of BBS-1 on the Expression of NOS Activity and Protein Dimerization. The validated adenovirus expression system in DLD-1 cells was used to examine the effects of BBS-1 on the assembly of active nNOS. As shown in Fig. 2A, increasing concentrations of BBS-1 added to cells 2 h after viral infection led to a concentration-dependent decrease in L-arginine conversion (measured 24 h after infection). The apparent IC_{50} of BBS-1 was 40 ± 10 nM in intact cells. Similarly, treatment of DLD-1 cells with BBS-1 caused a decrease of accumulated NOS activity measured directly in the cell lysates after 24 h (IC_{50} = 74 ± 18 nM; data not shown). In contrast, the activity of purified rat nNOS was hardly affected at all by BBS-1 (IC_{50} ~1 mM, Fig. 2B).

Based on previous studies suggesting that BBS-1 interferes with iNOS dimerization, we monitored monomer/dimer ratios of human nNOS overexpressed in DLD-1 cells in the presence of increasing concentrations of BBS-1. As shown for porcine (Klatt et al., 1995) and rat (Klatt et al., 1996b) nNOS, the human enzyme partially retained its dimeric structure in the course of denaturating polyacrylamide gel electrophoresis. In the absence of BBS-1, the apparent dimer content was ~35%. Notably, this value represents not dimer content of nNOS under native conditions but survival of dimers in the presence of SDS (dimer stability). It cannot be excluded that the actual dimer content is underestimated by this method because of the comparably low blotting transfer efficiency of 320-kDa dimers. As shown in Fig. 3, presence of BBS-1 during de novo expression of human nNOS led to a concentration-dependent decrease in the relative amount of the dimeric protein without significant effects on total NOS protein level. Quantitative analysis of band intensities revealed an IC_{50} of 22 ± 5.2 nM BBS-1. This value is in excellent agreement with the apparent IC_{50} for inhibition of NOS activity in intact cells (40 nM, see Fig. 2A), suggesting that the drug does indeed act through interference with nNOS dimerization.

This conclusion was further confirmed by experiments designed to study the effect of BBS-1 added to the infected cells at later time points. In the absence of the inhibitor, NOS

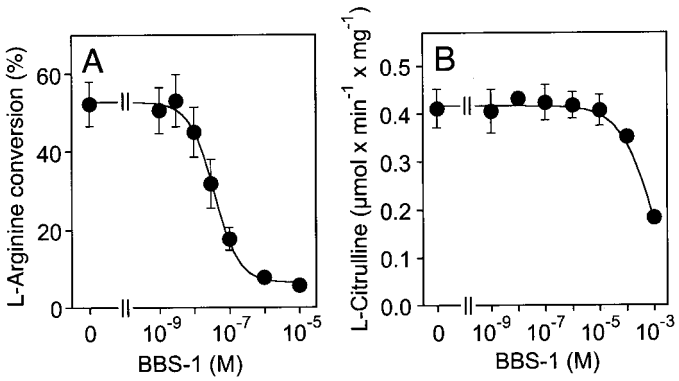

Fig. 2. Inhibition of nNOS by BBS-1. A, DLD-1 cells were infected with hnNOS-Ad (15 μl) for 24 h in the presence of increasing concentrations of BBS-1 added 2 h after infection, followed by determination of [^{3}H]arginine-to-[^{3}H]citrulline conversion by the intact cells stimulated with A23,187 (0.3 μM). B, activity of rat nNOS purified from Sf9 cells determined under standard conditions (see *Materials and Methods*) in the presence of increasing concentrations of BBS-1. Data are mean values ± S.E.M. of six (A) or two (B) experiments.

activity in intact cells became detectable 6 h after infection and increased to a maximum after 20 to 24 h. As expected for an inhibitor of protein assembly, excess BBS-1 (10 μM) prevented the further increase in activity when added to the cells 2, 7 or 12 h after infection (Fig. 4A). Based on the essential role of BH_4 for nNOS dimer stability (Klatt et al., 1995), these experiments were repeated with BH_4-depeleted cells. For this purpose, nNOS was expressed in DLD-1 cells that were pretreated with an inhibitor of GTP cyclohydrolase I, the rate-limiting enzyme in BH_4 synthesis (Werner et al., 1998). Preincubation of DLD-1 cells with 10 mM DAHP for 48 h led to an approximately 10-fold decrease in cellular BH_4 levels (from 7.2 ± 0.2 to 0.75 ± 0.04 pmol BH_4/mg of protein) that was accompanied by a significant decrease of L-arginine conversion (from 48 to 27%). However, the time course of de novo NOS expression with limiting BH_4 availability was similar to that observed in untreated cells; again, addition of excess BBS-1 (10 μM) completely blocked the further increase in expression of NOS activity (Fig. 4B).

The effects of BBS-1 on uncoupled NADPH oxidation catalyzed by nNOS in the absence of L-arginine or BH_4 were studied with the human enzyme purified from DLD-1 cells that had been infected with the adenovirus in the absence and presence of 10 μM BBS-1. As shown in Table 1, the specific activity of purified human nNOS expressed under control conditions (588 ± 18 nmol/min/mg) was similar to that of the rat brain enzyme obtained from a baculovirus overexpression system (Harteneck et al., 1994). Formation of H_2O_2, determined in the absence of L-arginine and BH_4 as a measure of uncoupled NADPH oxidation, was 711 ± 15 nmol/min/mg and thus markedly higher than the corresponding activity of rat nNOS (243 ± 20 nmol/min/mg). As expected from previous results with porcine (Heinzel et al., 1992) and rat brain (Gorren et al., 1996) NOS, H_2O_2 formation was virtually abolished in the presence of L-arginine and BH_4, and L-NNA (1 mM) inhibited formation of both L-citrulline and H_2O_2 by >90%. The enzyme expressed in the presence of BBS-1 was completely inactive in terms of L-citrulline formation; H_2O_2 formation was very low (1.4% of control) but above background. This residual uncoupled activity was insensitive to L-NNA (Tab. 1).

It was of interest to study whether the interaction of nNOS with BBS-1 is reversible. To account for possible recovery of enzyme activity after wash-out of the inhibitor because of de novo synthesis of active NOS, DLD-1 cells were infected for 20 h in the presence of excess BBS-1 (10 μM), followed by incubation of the cells in BBS-1-free medium with and without cycloheximide, a well-established inhibitor of protein expression (Obrig et al., 1971). As shown in Fig. 5, NOS activity recovered with a half-time of approximately 2 h upon wash-out of BBS-1 and was almost back to control values (determined with infected DLD-1 cells not exposed to the inhibitor) after 8 h. Because inhibition of protein synthesis by cycloheximide had no significant effect, these results suggest that BBS-1–triggered accumulation of nNOS monomers is fully reversible in intact cells.

Long-Term Effects of BBS-1 on Constitutively Expressed nNOS and eNOS. The observation that BBS-1 did not inhibit purified nNOS (Fig. 2B) or the active enzyme expressed in DLD-1 cells (Fig. 4) does not exclude the possibility that constitutively expressed NOS becomes affected upon long-term exposure to the dimerization inhibitor. To address this, we used HEK 293 cells stably transfected with

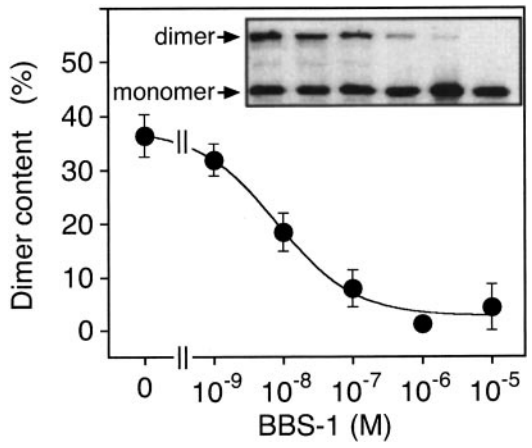

Fig. 3. Effect of BBS-1 on nNOS monomer/dimer equilibrium measured by LT-PAGE. DLD-1 cells were infected with hnNOS-Ad (15 μl) for 24 h in the presence of increasing concentrations of BBS-1 added 2 h after infection, followed by LT-PAGE/immunoblotting analysis of the cell lysates. The relative amount of nNOS dimers was quantified by densitometric analysis of band intensities (see *Materials and Methods* for details). The insert shows one of six similar blots (the six lanes from left to right refer to the BBS-1 concentrations indicated in the *x*-axis of the summary graph). No nNOS signal was observed in untransfected cells (not shown). Summary data are mean values ± S.E.M. of 6 blots.

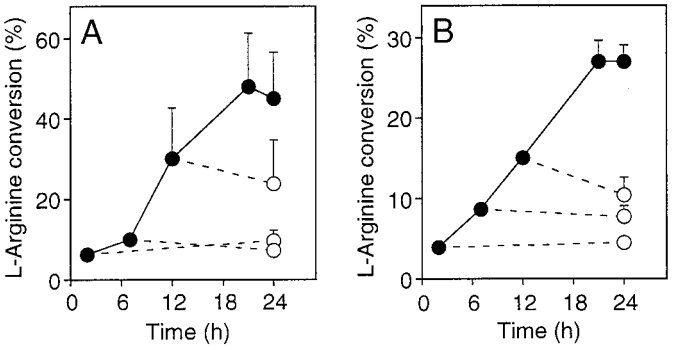

Fig. 4. Effect of time of BBS-1 addition on expression of nNOS activity in DLD-1 cells infected under control conditions (A) and after preincubation for 48 h with the inhibitor of BH_4 synthesis, DAHP (10 mM) (B). NOS activity was measured as [^{3}H]arginine-to-[^{3}H]citrulline conversion by the intact cells stimulated with A23,187 (0.3 μM) 2, 7, 12, 21, and 24 h after infection (filled symbols). In separate experiments BBS-1 (10 μM) was added 2, 7, or 12 h after the virus solution (origins of the dotted lines), followed by the determination of NOS activity 24 h after infection (open symbols). Data are mean values ± S.E.M. of three experiments.

TABLE 1

Characterization of human nNOS purified from DLD-1 cells infected for 40 h with hnNOS-Ad in the absence or presence of 10 μM BBS-1 and compared with purified rat nNOS obtained from baculovirus-infected Sf9 cells

Formation of L-citrulline and H_2O_2 was measured as described under *Materials and Methods*. L-Arginine and BH_4 were present at 1 and 0.2 mM, respectively. Unless indicated otherwise, data are mean values ± S.E.M. of two experiments.

	Control	10 μM BBS-1	Purified Rat nNOS
		nmol/min/mg	
L-Citrulline formation	588 ± 18	N.D.	615 ± 5
+ L-NNA (1 mM)	26 ± 20	N.D.	6 ± 3.6
H_2O_2 formation	711 ± 15	10 ± 7	243 ± 20
+ L-NNA (1 mM)	49 ± 2	10 ± 2	16 ± 4
+ L-Arginine, + BH_4	1 (n = 1)	N.D.	27 (n = 1)

N.D., not determined.

human nNOS or eNOS (Schmidt et al., 2001). Incubation of the cells with BBS-1 for up to 72 h led to a time-dependent decrease of cellular NOS activity (Fig. 6, A and B). Fitting the data obtained with excess BBS-1 (100 μM) to first-order kinetics revealed a half-life of 30 $\pm$ 7.1 and 43 $\pm$ 3.3 h for the decrease in the activity of nNOS and eNOS, respectively. The concentration dependence of this effect was measured with transfected HEK 293 cells incubated for 72 h in the presence of increasing concentrations of BBS-1. These experiments revealed IC_{50} values of 0.21 $\pm$ 0.03 and 12 $\pm$ 0.5 μM BBS-1 for inhibition of nNOS and eNOS, respectively (Fig. 6, C and D). In cultured endothelial cells, which express markedly lower levels of eNOS than the transfected HEK cells, BBS-1 led to a time- and concentration-dependent decrease of eNOS activity with an IC_{50} of 17 $\pm$ 6.2 μM (measured 72 h after addition of the inhibitor; data not shown).

Discussion

Infection of DLD-1 cells with recombinant adenovirus was used as a high level de novo expression system for human nNOS. Based on the enzyme activity measured in cell lysates, expression levels were significantly higher than in established rat or human neuronal cell lines but still 10-to 20-fold lower than in baculovirus-infected Sf9 cells (Harteneck et al., 1994). Nevertheless, the system allowed the determination of NOS activity in intact cells and purification of small amounts of the recombinant protein for further characterization. Expressed human nNOS had expected properties with respect to NO synthesis, cofactor sensitivity, inhibition by L-NNA, immunoreactivity, and dimer stability.

The adenovirus system was used to study BBS-1, a novel iNOS dimerization inhibitor with reactivity to nNOS (McMillan et al., 2000; Blasko et al., 2002). We found that BBS-1 inhibited adenovirus-mediated de novo expression of nNOS activity in DLD-1 cells with a fairly low IC_{50} of ~40 nM. The following evidence suggests that inhibition was caused by interference with nNOS dimerization: 1) delayed addition of BBS-1 prevented further increases in nNOS activity without affecting activity already expressed; 2) BBS-1 had no effect on purified nNOS; 3) BBS-1 caused complete loss of SDS-resistant nNOS dimers; 4) BBS-1 had no effect on total NOS expression levels, ruling out transcriptional/translational effects. Based on the model proposed for interaction of iNOS with BBS-1 and related compounds (Blasko et al., 2002), our results suggest that BBS-1 prevents nNOS dimerization by forming a high affinity monomer-heme-inhibitor complex that cannot dimerize. According to this model, BBS-1 and related drugs do not interfere with binding of heme to nNOS monomers (i.e., the first step in protein dimerization) (Klatt et al., 1996b; Hemmens et al., 1998; Bender et al., 2000), but prevent the assembly of heme containing monomers to form active dimers.

To characterize expressed human nNOS, protein (~20 μg from 10 Petri dishes) was purified to near homogeneity from DLD-1 cells. The enzyme from control cells had a specific NO synthesis activity similar to rat nNOS, but uncoupled NADPH oxidation, measured as formation of H_2O_2 in the absence of L-arginine and BH_4 (Heinzel et al., 1992), was 3-fold higher than the activity of the rat brain enzyme. Human nNOS expressed in *Pichia pastoris* system also exhibits high uncoupled activity (up to 1 μmol/min/mg; K. Schmidt and B. Mayer, unpublished observations), indicating an intrinsic difference between rat and human nNOS. Character-

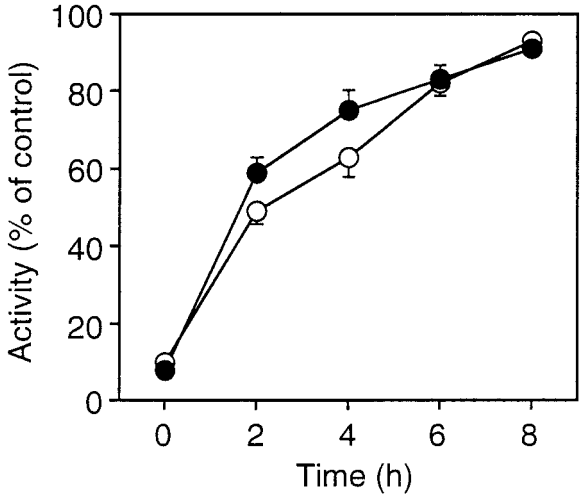

Fig. 5. Time-dependent recovery of NOS activity in DLD-1 cells upon wash-out of BBS-1. BBS-1 (10 μM) was added 2 h after hnNOS-Ad (15 μl). Twenty hours after virus infection, cells were washed and incubated in the presence of BBS-1–free medium with (filled symbols) or without (open symbols) 10 μM cycloheximide. At the indicated time points, NOS activity was determined as [^{3}H]arginine-to-[^{3}H]citrulline conversion by the intact cells stimulated with A23,187 (0.3 μM). Results are expressed relative to the activity of control cells not exposed to BBS-1. Data are mean values $\pm$ S.E.M. of three experiments.

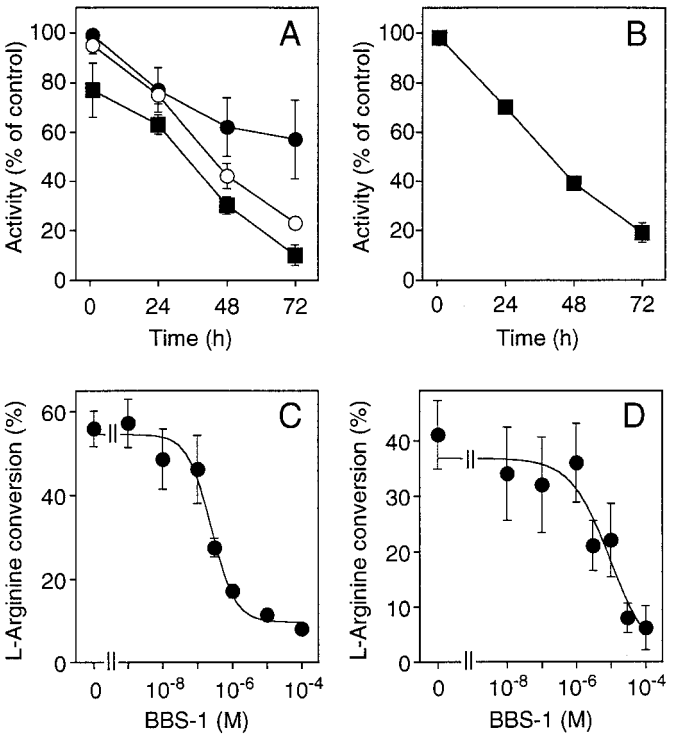

Fig. 6. Effect of BBS-1 on constitutively expressed NOS activity in HEK 293 cells. HEK 293 stably transfected with human nNOS (A) or human eNOS (B). Cells were incubated with buffer (100%) or with 0.1 μM (●), 10 μM (○), and 100 μM (■) BBS-1. At the indicated time points (1, 24, 48, and 72 h after addition of BBS-1), NOS activity was determined as [^{3}H]arginine-to-[^{3}H]citrulline conversion by the intact cells stimulated with A23,187 (0.3 μM). C and D, HEK 293 cells transfected with human nNOS (C) or human eNOS (D) were incubated for 72 h with the indicated concentrations of BBS-1, followed by determination of NOS activity as [^{3}H]arginine-to-[^{3}H]citrulline conversion by A23,187-stimulated cells. Data are mean values $\pm$ S.E.M. of three to six experiments.

ization of isolated nNOS from BBS-1–treated cells showed that the inhibitor-bound monomers were inactive in terms of both heme-catalyzed L-citrulline and uncoupled H_2O_2 formation. Residual uncoupled activity of this protein species was not L-NNA-sensitive, suggesting the involvement of the reductase rather than the oxygenase domain. The inability of BBS-1–bound monomers to catalyze O_2 reduction suggests that NADPH cannot reduce the heme of this protein species. This is consistent with the essential role of homodimerization for *in-trans* electron transfer (Siddhanta et al., 1998) and ligation of the monomer heme by the inhibitor (McMillan et al., 2000; Blasko et al., 2002). This is an important finding because it shows that NOS dimerization inhibitors result in the accumulation of monomers that cannot make NO and also cannot make oxygen-centered free radicals (H_2O_2 or superoxide) via the oxygenase domain. Both nNOS and iNOS can serve as sources of these damaging free radicals, particularly under conditions of substrate or pterin depletion (Heinzel et al., 1992; Pou et al., 1992; Xia and Zweier, 1997).

Binding of BBS-1 to iNOS monomers seems L-arginine- and BH_4-independent (Blasko et al., 2002). Neither substrate nor pterin is required for nNOS dimerization, but these agents stabilize the dimer (Klatt et al., 1995). BH_4 binding to nNOS dimers is anticooperative: the first pterin binds with subnanomolar affinity to one subunit, whereas micromolar BH_4 concentrations are required to saturate the second binding site (Klatt et al., 1994; Gorren et al., 1996; List et al., 1996; Alderton et al., 1998). These studies also suggested that high-affinity binding of one BH_4 molecule is sufficient for formation of stable dimers that generate NO/superoxide in a 1:1 stoichiometry. In line with this concept, NOS activity in ~10-fold BH_4-depleted DLD-1 cells was approximately half of control (see Fig. 4), whereas monomer/dimer ratios in LT-PAGE were not affected (data not shown). BH_4 depletion of DLD-1 cells had no effect on the action profile of BBS-1, suggesting it blocked nNOS dimerization in a pterin-independent manner.

BBS-1 binds reversibly to iNOS monomers (Blasko et al., 2002) with a slow off-rate ($\tau_{1/2} \sim 140$ min) as principal determinant of high affinity ($K_d = 2.2 \times 10^{-9}$ M). The number of binding sites approximates heme content (~10%), indicating that bound heme is essential for BBS-1 binding. It would be interesting to perform a similar study with nNOS. However, nNOS monomers obtained from heme-deficient Sf9 cells are essentially heme-free (Klatt et al., 1996b) and urea treatment of nNOS results in complete loss of heme (P. Klatt and B. Mayer, unpublished observations). As an alternative approach, reversibility of BBS-1 binding to nNOS was studied in cells. Human nNOS activity was undetectable when nNOS was expressed in the presence of excess BBS-1 (10 μM) but completely recovered with a half-time of 2 h after inhibitor wash-out. This value approximates the rate of BBS-1 dissociation from purified iNOS monomers (~140 min; Blasko et al., 2002). Faster dissociation might have been expected based on the 5-fold nNOS/iNOS selectivity of BBS-1 (McMillan et al., 2000), but recovery of enzyme activity in intact cells presumably reflects the rates of both BBS-1 dissociation and protein dimerization. In vitro, dimerization of nNOS (Bender et al., 2000) and iNOS (Blasko et al., 2002) monomers is a relatively slow process ($\tau_{1/2}$ ~30 min). If also true for NOS dimer assembly in intact cells, the rate of BBS-1 dissociation may be underestimated in cell-based assays. In any case, our results clearly demonstrate that BBS-1 acts as reversible inhibitor of nNOS dimerization in intact cells.

The lack of effect of BBS-1 on dimeric rat and human nNOS suggests little if any reverse equilibrium from nNOS dimers to monomers in vitro or in intact cells. Although nNOS inhibition by BBS-1 may be confined to the time of protein synthesis, the constitutively expressed isoforms may become inhibited upon long-term exposure to dimerization inhibitors, depending on their turnover rates. The results with constitutively expressed human nNOS and eNOS suggest that is indeed the case. Incubation of HEK 293 cells with BBS-1 led to a time-dependent decrease in NOS activity, with half-lives of 30 and 43 h for nNOS and eNOS, respectively. The apparent IC_{50} to inhibit nNOS and eNOS (0.21 ± 0.03 μM and 12 ± 0.5 μM, respectively) were in fairly good agreement with published selectivity data (McMillan et al., 2000). Inhibition of constitutively expressed NOS by BBS-1 is probably caused by blocked assembly of newly synthesized protein during steady-state turnover (i.e., reflecting NOS protein degradation in HEK cells). In other studies, [^{35}S]methionine labeling studies yielded higher rates of NOS turnover [$\tau_{1/2}$, 10–20 h (Noguchi et al., 2000; Ying et al., 2001)]. Relatively high NOS expression levels or lack of distinct proteolytic pathways in HEK cells may explain this difference. However, BBS-1 inhibited eNOS in endothelial cells with very similar time course and concentration dependence, ruling out distinct properties of the HEK 293 cell overexpression system to explain the slow rate of inhibition. Alternatively, the assembly of nNOS and eNOS dimers may not be fully inhibited even at high concentrations of BBS-1 (tested at 0.1 mM) or the drug could act as inhibitor of cellular NOS degradation pathways. Additional studies are needed to clarify this issue.

In summary, we provide convincing evidence that BBS-1 is a relatively potent and reversible inhibitor of de novo nNOS dimerization in intact cells and that this novel inhibitor can be used to study the process of NOS dimer assembly and turnover in a cellular context. The results with constitutively expressed nNOS and eNOS confirm the relatively slow turnover rate of these enzymes in cells and the weak and slow onset of action of the NOS dimerization inhibitor against these isoforms. These findings have important therapeutic implications. BBS-1 was selected as a tool compound to study nNOS dimerization based on its reported low nNOS/iNOS selectivity ratio (5-fold) in dimerization assays (McMillan et al., 2000). However, related compounds with optimized nNOS/iNOS ratios (> 600) that retain the high eNOS/iNOS selectivity of BBS-1 (> 1000) have been identified (J. F. Parkinson and G. B. Phillips, in preparation). Based on our present studies, these potent and selective iNOS dimerization inhibitors would be anticipated to inhibit the de novo assembly of iNOS in vivo under conditions of active cytokine-stimulated iNOS expression at steady-state plasma drug levels in the nanomolar range. In contrast, much higher steady-state plasma drug concentrations ($\gg$10 μM) would need to be sustained to exert any appreciable pharmacological effect on constitutive nNOS and eNOS activity. Effective in vivo iNOS selectivity would thus derive from weak intrinsic affinity of compounds for the nNOS and eNOS monomers, the relative stability of the eNOS and nNOS versus iNOS dimers (Panda et al., 2002), and the slow turnover of constitutively expressed eNOS and nNOS in cells and tissues. Dimerization

inhibitors thus provide a novel and highly selective strategy toward therapeutic iNOS inhibition in acute and chronic inflammatory disorders.

Acknowledgments

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