Tetrahydrobiopterin Protects against Guanabenz-Mediated Inhibition of Neuronal Nitric-Oxide Synthase in Vitro and in Vivo

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Received February 22, 2006; accepted May 22, 2006

ABSTRACT:

It is established that guanabenz inhibits neuronal nitric-oxide (NO) synthase (nNOS) and causes the enhanced proteasomal degradation of nNOS in vivo. Although the time- and NADPH-dependent inhibition of nNOS has been reported in studies where guanabenz was incubated with crude cytosolic preparations of nNOS, the exact mechanism for inhibition is not known. Moreover, even less is known about how the inhibition of nNOS triggers its proteasomal degradation. In the current study, we show, with the use of purified nNOS, that guanabenz treatment leads to the oxidation of tetrahydrobiopterin and formation of a pterin-depleted nNOS, which is not able to form NO. With the use of 14C-labeled guanabenz, we were unable to detect any guanabenz metabolites or guanabenz-nNOS adducts, indicating that reactive intermediates of guanabenz probably do not play a role in the inhibition. Superoxide dismutase, however, prevents the guanabenz-mediated oxidation of tetrahydrobiopterin and inhibition of nNOS, suggesting the role of superoxide as an intermediate. Studies in rats show that administration of tetrahydrobiopterin prevents the inhibition and loss of penile nNOS due to guanabenz, indicating that the loss of tetrahydrobiopterin plays a major role in the effects of guanabenz in vivo. Our findings are consistent with the destabilization and enhanced degradation of nNOS found after tetrahydrobiopterin depletion. These studies suggest that drug-mediated destabilization and subsequent enhanced degradation of protein targets will likely be an important toxicological consideration.

Nitrilotriacetic synthase (NOS) plays a key role in a variety of physiological processes, including neurotransmission and penile erection (Moncada et al., 1991; Burnett et al., 1992). Clinical experience and several publications have linked prescribed drugs with sexual dysfunction (Slag et al., 1983; Brock and Lue, 1993). The antihypertensive agents, in particular, are commonly associated with drug-induced impotence (Brock and Lue, 1993). Guanabenz, an antihypertensive agent associated with impotence (Weiss, 1991; Brock and Lue, 1993), inhibits NOS activity in penile tissue (Nakatsuka et al., 1998). Consistent with this finding, guanabenz inhibits NOS and enhances the proteasomal degradation of the enzyme in human embryonic kidney 293 cells (Noguchi et al., 2000). Guanabenz causes the time- and NADPH-dependent inhibition of NOS in an in vitro system containing penile cytosol (Nakatsuka et al., 1998; Noguchi et al., 2000). It is noteworthy that other time- and NADPH-dependent inhibitors of NOS, such as N′-methyl-L-arginine and N′-(1-iminoethyl)-L-ornithine, also enhance the proteasomal degradation of the enzyme in cells (Noguchi et al., 2000). The trigger is not due to the activity loss per se, since reversible inhibitors, such as N′-nitro-L-arginine and 7-nitroindazole, do not enhance degradation of nNOS and may actually stabilize the protein (Nakatsuka et al., 1998; Noguchi et al., 2000).

We wondered how the time-dependent inhibition of NOS renders the enzyme susceptible for degradation. Although guanabenz is well characterized with respect to degradation of NOS in cells and in rats, relatively little is known about how guanabenz inhibits NOS. In the current study, we chose to address this question with the use of purified NOS in the hope of understanding what dysfunctional forms of NOS are recognized for degradation. We found that guanabenz causes a tetrahydrobiopterin (BH4)-deficient state of NOS because of the oxidative destruction of the pterin that is facilitated by the presence of NADPH. The addition of BH4 completely reactivates this...
dysfunctional form of the enzyme. The administration of BH4 to rats completely protects from guanabenz-mediated inhibition of nNOS as well as the loss of nNOS protein, suggesting that the pterin deficiency plays a major role in the in vivo effects of guanabenz on nNOS.

**Materials and Methods**

**Materials.** Guanabenz was purchased from Research Biochemicals International (Natick, MA), Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADP+, N5-nitro-o-arginine, L-arginine, L-nicotine, dihydropteridine reductase from sheep liver, calmodulin, catalase, superoxide dismutase, and NADPH were purchased from Sigma Aldrich (St. Louis, MO). Male Wistar rats were purchased from Charles River Laboratories (Wilmington, MA). (6S)-5,6,7,8-Tetrahydro-L-biopterin (BH2) was purchased from Dr. Schirk’s (Jona, Switzerland). The affinity-purified rabbit IgG against brain NOS used for immunoblotting nNOS was from BD Biosciences Transduction Laboratories (Lexington, KY). [Benzyldiene carbon 14C]-labeled guanabenz (56 mCi/mmol) was custom-synthesized by Du Pont NEN (Boston, MA). L-[14C]-(U)-Arginine (330.0 mCi/mmol) and 125I-labeled antibody against rabbit IgG were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

**Methods.** *In vitro inhibition assays.* For studies on the inactivation of purified nNOS, we overexpressed the enzyme in insect cells and purified the nNOS as described previously (Billecke et al., 2004). Purified nNOS (80 μg/ml) was added to a “first reaction mixture” of 40 mM potassium phosphate, pH 7.4, containing 0.2 mM CaCl2, 2500 units/ml superoxide dismutase, 1250 units/ml catalase, 20 μg/ml pure calmodulin, 0.23 mg/ml bovine serum albumin, and an NADP+-regenerating system composed of 0.4 mM NADP+, 10 mM glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase/ml, expressed as final concentrations, in a total volume of 180 μl. After incubation at 30°C, aliquots (10 μl) of the first reaction mixture were transferred to an “oxyhemoglobin assay mixture” containing 200 μM CaCl2, 250 μM L-arginine, 100 units/ml catalase, 10 μg/ml crude calmodulin, 25 μM oxyhemoglobin, and the NADPH-regenerating system, in a total volume of 180 μl of 40 mM potassium phosphate, pH 7.4. The oxyhemoglobin assay mixture was incubated at 37°C, and the rate of NO-mediated oxidation of oxyhemoglobin was monitored by measuring the absorbance at 404 to 411 nm with a microtiter plate reader (SpectraMax Plus; Molecular Devices Corp., Sunnyvale, CA). The rate was determined from the linear portion of the time-dependent changes in absorbance. In studies where endothelial NOS was used, the enzyme was overexpressed and purified as described previously (Lowe et al., 2005). The assay conditions were the same as nNOS, except that 200 μg/ml of the endothelial NOS was used in the first reaction mixture and 20-μl aliquots were taken for the oxyhemoglobin assay mixture.

**SDS-resistant dimer analysis.** In studies where the SDS-resistant dimer was measured, we examined the samples by low temperature SDS-PAGE (Klett et al., 1995). nNOS forms a very tight dimer that is resistant to SDS at low temperatures. By keeping the samples on ice and running the SDS-PAGE with a cooling unit, the stable dimeric species can be visualized. In these studies, an aliquot (10 μl) of the first reaction mixture containing purified nNOS was quenched with an equal volume of sample buffer containing 5% SDS, 20% glycerol, 100 mM dithiothreitol, 200 μM L-arginine, and 0.02% bromphenol blue in 125 mM Tris-HCl, pH 6.8. The samples were kept on ice, and 10 μl of the quenched sample was loaded for analysis by SDS-PAGE. Proteins were then transferred to nitrocellulose membranes (0.2 μm; Bio-Rad, Hercules, CA) and probed with 0.1% anti-nNOS. The immunoblots were then incubated a second time with 125I-conjugated goat anti-rabbit IgGs to visualize the immunoreactive bands. The membranes were dried and exposed to X-OMAT film (Kodak, Rochester, NY) for 1 h at −80°C. The nitrocellulose bands corresponding to nNOS were excised and the radioactivity quantified by the use of a gamma counter.

**HPLC analysis.** The alteration of the heme prosthetic group was measured by HPLC similar to that described previously (Jianmogkol et al., 2000). HPLC was performed with the use of a Waters 600S controller, 717 plus autosampler, and 996 photodiode array detector (Waters Corp., Milford, MA). Samples were injected onto a reverse phase HPLC column (C4 Vydac, 5 μm, 0.21 × 15 cm; Grace Vydac, Hesperia, CA) equilibrated with solvent A (0.1% trifluoroacetic acid) at a flow rate of 0.3 ml/min. A linear gradient to 75 and 100% solvent B (0.1% trifluoroacetic acid in acetoniitrile) was run over 30 and 5 min, respectively. Absorbance at 220 and 400 nm was monitored.

**Quantification of BH4 and BH2.** The amounts of BH4 and 7,8-dihydrobipterin (BH2) in the reaction mixtures were determined by use of an HPLC fluorescence method as described by Klett et al. (1996). The method involves oxidation of BH4 and BH2 to bipterin by treatment with KI/I2 solution under acidic conditions. To give the specific amount of BHI, the KI/I2 oxidation is done in a basic solution where BHI and BHI2 are oxidized to pterin and bipterin, respectively. Specifically for oxidation under acidic conditions, a 40-μl aliquot of the first reaction mixture was treated with 10 mM I2 and 50 mM KI in a total volume of 50 μl of 100 mM HCl for 1 h at room temperature in the dark. The solution was neutralized with 5 μl of 1.0 M NaOH, and then 5 μl of 0.2 M ascorbate was added. An aliquot (30 μl) of the resulting solution was injected onto a reverse phase HPLC column (C18 Vydac, 5 mm, 4.6 × 250 mm) equilibrated with 20 mM NaH2PO4, pH 3, with 5% methanol at a flow rate of 1 ml/min. The ptetins were eluted with the same mobile phase and detected by fluorescence at excitation and emission wavelengths of 350 and 418 nm, respectively. The HPLC and analysis of ptetins was performed with the use of a Waters systems described above and an Applied Biosystems Spectroflow 980 fluorescence detector (Applied Biosystems, Foster City, CA). To oxidize the ptetins under basic conditions, the first reaction mixture was treated as above except that 100 mM NaOH replaced 100 mM HCl, and the final solution was neutralized with 5 μl of 1 M HCl.

**Treatment of nNOS with 125I-guanabenz.** The purified nNOS was treated as described above in a first reaction mixture, except that nNOS (1.5 μM) was treated with 50 μM guanabenz (56 mCi/mmol) for 60 min at 22°C. An aliquot (75 μl) was injected onto a reverse phase HPLC column (C4 Vydac, 5 μm, 2.1 × 150 mm) equilibrated with solvent A (0.1% trifluoroacetic acid) at a flow rate of 0.3 ml/min. A linear gradient to 75% solvent B (0.1% trifluoroacetic acid in acetoniitrile) was run over 45 min, and then a linear gradient to 100% B was run over the next 5 min. The absorbance at 220 nm was monitored. The radioactivity in the eluent was measured by an on-line radiochemical detector (Radiomatic 500TR; Packard, Dohners Grove, IL).

**Treatment of animals, sample preparation, and activity assays.** Guanabenz was dissolved in physiological saline and administered to male Wistar rats (150–250 g) at the indicated doses by intraperitoneal injection at 9:00 AM and 6:00 PM. BH4 was dissolved in 0.1% (w/v) ascorbic acid in physiological saline and injected in a total volume of 1 ml, 30 min before the injection of guanabenz. The controls were given the appropriate volumes of physiological saline or 0.1% (w/v) ascorbic acid in physiological saline. Rats were sacrificed by decapitation 16 h after the last injection. Whole deskinned penis was removed, washed with ice-cold physiological saline, cut into 1- to 2-mm pieces, and homogenized in 1 ml of ice-cold homogenization buffer (10 mM HEPES, pH 7.5, containing 320 mM sucrose, 100 μM EDTA, 1.5 mM dithiothreitol, 10 μg/ml trypsin inhibitor, 10 mg/ml leupeptin, 2 μg/ml apro tin, 1 mg/ml phenylmethylsulphonyl fluoride, and 100 μM BHI2) with the use of a glass homogenizer (Tekmar, Cincinnati, OH). The homogenate was centrifuged at 245,000g for 10 min at 4°C. The supernatant fraction was collected and frozen in liquid nitrogen and stored at −80°C for later analysis. Protein concentration of these samples was determined by the method of Bradford (Bio-Rad), with the use of bovine serum albumin as a standard.

The NOS activity of samples from the in vivo studies were determined by adding the supernatant fraction (0.6 mg) to a “citrulline assay mixture” containing 1 mM CaCl2, 1 mM NADPH, 30 μM [14C]arginine (60 mCi/mmol), 100 μM BH4, and 10 μg/ml calmodulin in a total volume of 200 μl of 40 mM potassium phosphate, pH 7.4. The assay mixture was incubated at 37°C for 10 min, and the amount of [14C]citrulline was determined as described previously (Nakatsuka et al., 1998). The formation of [14C]citrulline was linear over the 10-min period. For experiments on the in vitro inactivation of cytosolic NOS, the supernatant fraction from untreated rats was loaded onto a Sephadex G-25 column (PD-10; Pharmacia Biotech, Piscataway, NJ) pre-equilibrated in 10 mM HEPES, pH 7.5, containing 320 mM sucrose, 100 μM EDTA, 1.5 mM dithiothreitol, 10 μg/ml trypsin inhibitor, 10 mg/ml leupeptin, 2 μg/ml aprotinin, and 1 mg/ml phenylmethylsulphonyl fluoride to remove endogenous arginine and excess BH4. An aliquot (1.2 mg) of the gel-filtered fraction was placed in a “reaction mixture” containing 1 mM CaCl2, 1 mM NADPH, 10 μg/ml calmodulin, and the desired concentration of guanabenz, in

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In vitro ubiquitylation of guanabenz-treated nNOS. We used an in vitro ubiquitylation system containing fraction II that has been established to ubiquitylate nNOS by an ATP-dependent process (Kamada et al., 2005). The nNOS was treated with 100 μM guanabenz as described above, and an aliquot (160 μl) of this first reaction mixture was incubated at 37°C in a total volume of 400 μl of 50 mM Tris-HCl, pH 7.4, containing 2 mM dithiothreitol, 10 μM ubiquitin, 15 μM ubiquitin, an ATP-regenerating system (2 mM ATP, 10 mM creatine phosphate, 5 mM MgCl2, and 10 units/ml creatine phosphokinasen), and 0.4 mg/ml fraction II. An aliquot (20 μl) of the samples was quenched with 20 μl of sample buffer containing 5% SDS, 20% glycerol, 100 mM dithiothreitol, and 0.02% bromphenol blue in 125 mM Tris-HCl, pH 6.8. The samples were boiled for 5 min, and an aliquot (30 μl) was submitted to 6% SDS-PAGE (10 × 8 cm). Proteins were then transferred to nitrocellulose membranes (0.2 μm, Bio-Rad) and probed with 0.5% anti-ubiquitin (DAKO, Carpinteria, CA). The immunoblots were then incubated a second time with 125I-conjugated goat anti-rabbit IgGs to visualize the immunoreactive bands. The membranes were dried and exposed to X-OMAT film (Kodak, Rochester, NY) as described by the manufacturer. The intensity of the nitrocellulose membrane (Schleicher and Schuell, Keene, NH) was used as a secondary antibody. An enhanced chemiluminescence reagent (Amersham Life Science Inc., Arlington Heights, IL) and X-OMAT film (Kodak, Rochester, NY) were used to detect the peroxidase conjugate, as described by the manufacturer. The intensity of the bands was evaluated by a laser densitometer (Molecular Dynamics, Sunnyvale, CA). Differing amounts of cytosol prepared from rat brains or insect cells overexpressing neuronal NOS were analyzed to ensure that the density was linearly dependent on the amount of NOS over the relevant concentration range.

Results

Guanabenz-Mediated Inhibition of Purified nNOS. It is established that guanabenz inhibits NOS and enhances the proteolytic turnover of NOS protein in cells (Noguchi et al., 2000). Consistent with this, administration of guanabenz to rats decreases NOS activity and protein (Nakatsuka et al., 1998; Dambrova et al., 2003). Moreover, the time-dependent inhibition of NOS due to guanabenz has been characterized in in vitro studies with the use of penile cytosol (Nakatsuka et al., 1998). To better understand the mechanism of how guanabenz inhibits NOS and causes the enhanced turnover of the enzyme, we chose to conduct studies with purified nNOS. We established here, for the first time, that guanabenz causes a time-dependent inhibition of purified nNOS (Fig. 1A, closed squares). There is a loss of activity even in the absence of guanabenz (open squares), albeit slower, representing an autoinactivation reaction. The half-life of the activity loss was 5.0 ± 1.8 and 22.1 ± 6.9 min for the guanabenz-treated and untreated samples, respectively. These values are statistically different (p < 0.05). The loss of NOS activity beyond this autoinactivation is dependent on the concentration of guanabenz (Fig. 1B). Taken together, these results are highly similar to those found when the nNOS in penile cytosol was treated with guanabenz (Nakatsuka et al., 1998). Moreover, guanabenz did not inhibit endothelial NOS, suggesting that the action of guanabenz is selective (Fig. 1B, inset). The autoinactivation of NOS in the absence of substrate or guanabenz is time-, calmodulin-, and NADPH-dependent (Demady et al., 2001). The autoinactivation could be due to alteration of critical amino acid residues, the prosthetic heme group, or tetrahydrobiopterin (Demady et al., 2001).

The time-dependent inhibition of activity is thought to produce a dysfunctional, altered form of NOS that is preferentially ubiquitylated and proteasomally degraded (Noguchi et al., 2000). Recently, it was found that destabilization of the dimeric functional form of NOS correlates with recognition for proteasomal degradation (Dunbar et al., 2004). Thus, we asked if guanabenz destabilizes NOS dimers. As shown in Fig. 2A, the untreated nNOS exists in part as SDS-resistant dimers, which are visualized after low-temperature SDS-PAGE and

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**Fig. 1.** Guanabenz-mediated inactivation of purified nNOS. A, time-dependent loss of nNOS activity as a result of guanabenz on a semi-log plot. Closed squares, treated with 100 μM guanabenz; open squares, untreated. The inactivation of nNOS activity was determined with the use of the modified first reaction mixture and oxyhemoglobin assay mixture as described in Materials and Methods. The half-life for each condition was calculated by least-squares fitting of the semi-log plot. *p < 0.05. **B**, the effect of varying the concentration of guanabenz in the first reaction mixture. The amount of guanabenz is indicated, and the activities were measured after 20 min of incubation. Inset, the endothelial NOS replaced nNOS, and the effects of guanabenz were determined. *p < 0.05 different from untreated. The values are the mean ± S.E. (n = 3).

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**Statistical analysis.** All values are reported as the mean ± standard error (S.E.). An unpaired t test was used to compare values. We considered statistical significance to be achieved at a level of p < 0.05. PRISM statistical software (GraphPad, San Diego, CA) was used for analysis of the data sets.
subsequent immunoblotting (Klatt et al., 1995). We quantified the bands corresponding to the dimer and monomer from these studies (Fig. 2B). This assay is not a measure of the dimeric content under native conditions but is a measure of the amount of stable dimer that is not dissociated by SDS and, thus, underestimates the total dimeric content. Moreover, it is likely that the transfer efficiency of the dimer is lower than that of the monomer, further leading to the underestimation of dimeric content. Thus, we cannot determine the absolute amounts of each form, but we can determine the relative changes in dimer and monomer.

As shown in Fig. 2B, we found that the SDS-resistant dimeric form of the untreated nNOS is unstable (closed circles), probably reflecting an autoinactivation reaction. Treatment with guanabenz further destabilizes the dimeric nNOS (closed squares) and gives an increase in the monomeric nNOS (open squares). As shown in Fig. 2C, the destabilization of the dimeric nNOS (closed triangles) and the formation of monomeric nNOS (open triangles) is dependent on the concentration of guanabenz. Both the time dependence and concentration dependence of the loss of dimeric nNOS reflect the loss of NOS activity seen above. These results are entirely consistent with the notion that stabilization of the dimer by guanabenz generates some altered NOS form that is more susceptible for proteasomal degradation. We next sought to determine how guanabenz destabilizes the nNOS dimer.

As shown in Fig. 3A, we first determined the cofactor dependence of the inhibition by treating nNOS under the indicated conditions for 20 min in the presence (open bars) or absence (closed bars) of 100 μM guanabenz. The greatest decrease in activity due to guanabenz occurs when both calmodulin and NADPH are present. Under these conditions, the L-isomer, but not the D-isomer, of arginine protects from the inhibition, suggesting an active site-directed process. These findings are similar to those found for nNOS in penile cytosol (Nakatsuka et al., 1998). However, unlike the previous observations with crude cytosol, approximately half the activity is lost even when purified nNOS is treated with guanabenz in the absence of exogenous NADPH. Even under this condition, calmodulin is necessary for guanabenz-mediated inhibition of nNOS (data not shown). We wondered why NADPH was necessary for inhibition of the crude cytosolic preparation of nNOS (Nakatsuka et al., 1998) but not for the purified enzyme. To better understand the inactivation process and why there are seemingly disparate findings, we further investigated both the mechanism of inactivation without NADPH as well as that found with NADPH. In the course of our studies, we discovered that the concentration of BH4 in the first reaction mixture is a critical factor and that the presence of NADPH, superoxide dismutase, and catalase also has an effect on the guanabenz-mediated inhibition of nNOS. To dissect these effects, we initially investigated the effect of BH4 on the guanabenz-mediated inhibition of nNOS in the absence of superoxide dismutase and catalase (Fig. 3B). Under these conditions, guanabenz in the presence of NADPH causes a nearly complete inactivation of

**FIG. 2.** Effect of guanabenz on the amount of SDS-resistant dimer of nNOS. The nNOS was treated with guanabenz as described under Materials and Methods, and the amount of the SDS-resistant dimer (nNOS dimer) and the remainder of the nNOS that runs as a monomer (nNOS monomer) were measured. A, Western blot of the reaction mixture of untreated nNOS (Untreated) or nNOS treated with guanabenz (Guanabenz) for 0, 15, 30, and 45 min. B, the bands corresponding to the nNOS dimer and monomer in A were quantified by the use of 125I-labeled goat anti-rabbit IgG. Circles, untreated; squares, guanabenz-treated. The dimeric nNOS is represented by the closed symbols and the solid lines, and the monomeric nNOS is represented by the open symbols and the dashed lines. *, significantly (p < 0.05) lower dimer for guanabenz-treated versus untreated. #, significantly (p < 0.05) higher monomer for guanabenz-treated over the untreated. C, the effect of varying the concentration of guanabenz on the amount of SDS-resistant dimer after treatment for 20 min. The bands were quantified as in B. Solid triangles, nNOS dimer; open triangles, monomeric nNOS. *, significantly (p < 0.05) lower dimer than untreated. #, significantly (p < 0.05) higher monomer than untreated. The values are the mean ± S.E. (n = 3).
nNOS, and the addition of BH$_4$ during the treatment has a protective effect (solid circles). Interestingly, NADPH alone has a large inhibitory effect, but in this case, the addition of even small amounts of BH$_4$ protects from the loss of activity (compare solid squares with open squares). Low levels of BH$_4$ also protect the enzyme when nNOS is treated with guanabenz in the absence of NADPH (open circles).

Overall, greater inhibition of nNOS is observed when NADPH and guanabenz are present over that when NADPH is omitted (compare solid circles with open circles). This clearly demonstrates how NADPH-dependent inactivation of nNOS could be observed depending on the BH$_4$ concentration, and this probably explains the seemingly disparate observation made in a previous study on NADPH dependence (Nakatsuka et al., 1998).

As shown in Fig. 3C, BH$_4$ completely protects the enzyme from inactivation when nNOS is treated with guanabenz and NADPH in the presence of superoxide dismutase and catalase (compare solid triangles with open triangles). In addition, the presence of superoxide dismutase and catalase protects against the autoinactivation of nNOS that occurs in the presence of NADPH. As a control, we show that BH$_4$ does not affect the inhibition of nNOS because of N$^\text{G}$-nitro-$l$-arginine, a slowly reversible active site-directed inhibitor ($X$). It is noteworthy that N$^\text{G}$-nitro-$l$-arginine stabilizes the SDS-resistant dimeric form of nNOS (Dunbar et al., 2004). Thus, guanabenz seems to inhibit nNOS by a process that is antagonized by BH$_4$. We will show below that a BH$_4$-deficient enzyme is formed.

Tetrahydrobiopterin Depletion As a Mechanism for Guanabenz-Mediated Inhibition of Purified nNOS. Initially, we investigated the guanabenz-mediated inhibition of nNOS in the absence of NADPH. As shown in Fig. 4A, the calmodulin- and guanabenz-dependent loss of activity occurs concomitantly with the loss of BH$_4$. Approximately 60% of the loss of BH$_4$ is accounted for by the formation of BH$_2$. As shown in Fig. 4B, the activity loss seen when nNOS is incubated with calmodulin and guanabenz in the absence of NADPH is completely reversed by the addition of BH$_4$ in the oxyhemoglobin assay mixture. A concentration of 0.1 $\mu$M BH$_4$ is sufficient for complete reversal, consistent with the approximate loss of BH$_4$. The restoration of activity is rapid, since there is no incubation step with BH$_4$ before the activity is measured. The restoration is nearly complete, suggesting that the depletion of BH$_4$ is the major mechanism for the activity loss under these conditions. It is noteworthy that in previous studies with the cytosolic fraction containing nNOS (Nakatsuka et al., 1998), BH$_4$ was present in the assay mixture; therefore, the NADPH-independent inhibition of nNOS by guanabenz would have been obscured. There is also a loss of BH$_4$ after inhibition...

![Fig. 3. Effect of substrate, NADPH, tetrahydrobiopterin, and calmodulin on the guanabenz-mediated inactivation of nNOS. A, the effect of calmodulin (CAM), NADPH, $l$-arginine ($l$-Arg), and $d$-arginine ($d$-Arg) on the extent of inhibition of nNOS after treatment for 20 min with 100 $\mu$M guanabenz (open bars) or untreated (solid bars). B, the protective effect of BH$_4$ on the inhibition of nNOS caused...](at ASPET Journals on June 25, 2017 dmd.aspetjournals.org Downloaded from)

![Fig. 4. Tetrahydrobiopterin Depletion As a Mechanism for Guanabenz-Mediated Inhibition of Purified nNOS. A, the calmodulin- and guanabenz-dependent loss of activity occurs concomitantly with the loss of BH$_4$. Approximately 60% of the loss of BH$_4$ is accounted for by the formation of BH$_2$. As shown in Fig. 4B, the activity loss seen when nNOS is incubated with calmodulin and guanabenz in the absence of NADPH is...](at ASPET Journals on June 25, 2017 dmd.aspetjournals.org Downloaded from)
of nNOS in the presence of NADPH (Fig. 4C). The addition of dihydropteridine reductase, which reduces BH2 to BH4, abolishes the inhibition of nNOS as a result of guanabenz (Fig. 4C), indicating that the oxidation of BH4 to BH2 and the subsequent formation of a BH4-deficient nNOS is the cause of the nNOS inhibition. Consistent with this finding, BH4 completely reverses the inhibition of nNOS even when NADPH is present (Fig. 4D). This is similar to that found above for the inhibition when NADPH was omitted. Thus, under all conditions examined here, BH4 deficiency is the major mechanism of nNOS inhibition due to guanabenz.

**Superoxide Dismutase Prevents the Guanabenz-Mediated Loss of nNOS Activity and Tetrahydrobiopterin.** A more detailed analysis of the dependence on catalase and SOD was performed (Fig. 5). As shown in Fig. 5A, superoxide dismutase protects against the loss of activity with nearly complete protection at 1000 units/ml (solid circles). Catalase at concentrations up to 400 units/ml has no effect (solid triangles). In a previous study, 100 units/ml catalase completely protected nNOS from the oxidative inactivation caused by agmatine (Demady et al., 2001). Superoxide dismutase alone is nearly as effective as superoxide dismutase in combination with catalase (solid squares). Thus, it seems that the nNOS-mediated superoxide formation is mainly responsible for the activity loss. Consistent with these findings, superoxide dismutase alone could completely protect against the guanabenz-mediated loss of BH4 (Fig. 5B).

**Studies with Radiolabeled Guanabenz.** The purified nNOS was treated with radiolabeled guanabenz to determine whether any adducts of guanabenz with nNOS or guanabenz metabolites could be detected. As shown in Fig. 6A, reverse phase HPLC analysis of the entire first reaction mixture containing nNOS and radiolabeled guanabenz but not calmodulin gives a major radiolabeled peak (solid line, G) corresponding to guanabenz. The peak at 37 min with absorbance at 220 nm corresponds to nNOS (dashed line, NOS). Treatment of nNOS with radiolabeled guanabenz in the presence of calmodulin did not cause an observable change in the radioactivity profile. We were thus unable to detect the metabolism of guanabenz. These results are consistent with the notion that BH4 deficiency is the major mechanism of nNOS inhibition.

**Tetrahydrobiopterin Protects against Guanabenz-Mediated Inhibition of Penile NOS Activity in Rats.** We sought to determine whether the inhibition and loss of nNOS protein in vivo could be ameliorated by BH4. We used a previously established procedure for treatment of rats with 5 mg/kg/day guanabenz for 4 days (Nakatsuka et al., 1998). As shown in Fig. 7A, guanabenz causes an approximately 50% reduction in NOS activity and nNOS protein in penile
tissue, highly similar to that described previously (Nakatsuwa et al., 1998). The concurrent administration of 200 mg/kg/day of BH4 completely abrogates the inhibitory effect of guanabenz and prevents the loss of nNOS protein. The administration of the same dose of BH4 alone has no effect on NOS activity or level of nNOS protein. The dose dependence of the protection by BH4 is shown in Fig. 7B. The amounts required to see an effect on nNOS are higher than those used in rats in previous studies on vascular function, which, for the most part, reflects endothelial NOS activity (Hong et al., 2001; Podjarny et al., 2004). The effects of BH4 on nNOS are not as well characterized, and we know of no studies in penile tissue. To further examine the role of BH4 on the guanabenz-mediated inactivation and protein turnover, we prepared desalted cytosol from penile tissue of untreated rats for use in in vitro inactivation studies. As shown in Fig. 7C, 100 μM guanabenz decreased nNOS activity by approximately one-half after treatment for 15 min (solid circles). We found that the addition of BH4 in the reaction mixture completely prevents the inactivation due to guanabenz, with a concentration of 1 μM giving nearly complete protection. The concentration dependence of BH4 found here is highly similar to the concentration dependence reported for the activation of nNOS in desalted rat cerebellar cytosol, where 1 μM BH4 was required for maximal activation of nNOS (Nunokawa et al., 1992). The addition of BH4 does not increase the activity of the untreated sample (X) nor protect against the inhibition by Nω-nitro-L-arginine (open circles), a slowly reversible active site-directed inhibitor. These results are highly similar to that found for the purified nNOS above.

**In Vitro Ubiquitylation of Guanabenz-Treated nNOS.** As shown in Fig. 8, top panel, there is a ubiquitin conjugate that is readily visualized with anti-ubiquitin in the 160-kDa region. The identity of this band as a nNOS-ubiquitin conjugate has been previously established (Bender et al., 2000; Kamada et al., 2005). The band corresponding to nNOS-ubiquitin was quantified and plotted (Fig. 8, bottom panel). There is an increase in the nNOS-ubiquitin conjugates found for guanabenz-treated nNOS (lane 2) over that for untreated nNOS (lane 1). When MG132, an inhibitor of the proteasome, is not present, then the nNOS-ubiquitin conjugate due to guanabenz is greatly reduced (lane 4).

**Discussion**

Guanabenz is known to enhance the proteosomal degradation of nNOS (Noguchi et al., 2000). It is thought that this labilization of the protein for degradation involves some alteration of the structure of nNOS, such that it is recognized by cellular factors that in turn lead to nNOS ubiquitylation and degradation. To better understand how guanabenz causes the selective removal of nNOS by the proteasome, we chose in the current study to determine how guanabenz alters nNOS. We found that guanabenz causes a destabilization of the native dimeric structure of the purified enzyme. This destabilization was because of the guanabenz-mediated, nNOS-catalyzed destruction of BH4, which stabilizes the active dimeric state of nNOS. Moreover, the treatment of rats with BH4 completely protects from the guanabenz-mediated inhibition and loss of penile nNOS. Although this suggests that the loss of BH4 is a mechanism for the inhibition and loss of nNOS in vivo, further studies on BH4 are needed to fully understand the molecular mechanisms responsible.

Superoxide dismutase, but not catalase, prevents the loss of BH4 from purified nNOS treated with guanabenz, indicating that nNOS-derived superoxide is responsible for the loss of BH4. The reaction of

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**Fig. 5.** Effect of superoxide dismutase on the guanabenz-mediated loss of nNOS activity and tetrahydrobiopterin. The nNOS was treated with guanabenz (100 μM) for 20 min, and the nNOS activity and pterin were measured as described under Materials and Methods. A, nNOS activity. The amount of superoxide dismutase (SOD) or catalase (CAT) or both were varied in the reaction mixtures treated with guanabenz. Closed squares, a combination of SOD and CAT were added; closed circles, SOD was added; closed triangles, CAT was added. As a control, SOD and CAT were added to a reaction mixture not treated with guanabenz (open squares). B, tetrahydrobiopterin. The 100 μM guanabenz (G), 2500 units/ml SOD, or 1 mM NADPH was omitted from the reaction mixtures as indicated. The values are the mean ± S.E. (n = 3). * Significantly (p < 0.05) lower values relative to untreated.

**Fig. 6.** HPLC profile of nNOS treated with 14C-labeled guanabenz in the absence or presence of calmodulin. The nNOS was treated with radiolabeled guanabenz as described under Materials and Methods. A, nNOS activity. The amount of superoxide dismutase (SOD) or catalase (CAT) or both were varied in the reaction mixtures treated with guanabenz. Closed squares, a combination of SOD and CAT were added; closed circles, SOD was added; closed triangles, CAT was added. As a control, SOD and CAT were added to a reaction mixture not treated with guanabenz (open squares). B, tetrahydrobiopterin.
superoxide with tetrahydrobiopterin has been previously reported and shown to form BH$_2$ (Vasquez-Vivar et al., 2001). Dihydropteridine reductase, which reduces BH$_2$ to BH$_4$, completely protects against the guanabenz-mediated inhibition of nNOS activity and loss of BH$_4$, strongly suggesting that BH$_4$ is oxidized to BH$_2$. However, under in vivo conditions where L-arginine is present, it seems that other reactive metabolites such as peroxynitrite, which can form by the reaction of superoxide with NO, are more likely the actual agents responsible for BH$_4$ oxidation (Milstien and Katusic, 1999; Laursen et al., 2001; Kuzkaya et al., 2003).

In the case of nNOS, BH$_4$ is known to stabilize the native dimeric state of the enzyme, and thus the oxidation of the BH$_4$ would destabilize the dimeric form, consistent with our findings. This may be important since destabilization of the dimer has recently been shown to lead to the ubiquitylation of nNOS in vitro (Dunbar et al., 2004) and in cells (Kamada et al., 2005). This notion is furthered by the finding that stabilization of the dimeric form of nNOS by NG-nitro-L-arginine or 7-nitroindazole protects nNOS from proteasomal degradation (Dunbar et al., 2004).

Although dimer stabilization plays an important role, the actual signal or recognition site for degradation is not clear. The trigger may be due to exposure of a site that is normally hidden in the active dimeric form of nNOS, a general unfolding of nNOS after perturbation of the BH$_4$ site, or exposure of hydrophobic residues in the heme-active site cleft (Peng et al., 2004). A recent report on the structure of a loose dimer of NOS with a partially exposed active
center and destabilized subdomains is entirely consistent with this view (Pant and Crane, 2005). The cellular factors that recognize the dysfunctional altered nNOS are also not known. In this respect, we have recently found that CHIP (C terminus of Hsc-70 interacting protein), a chaperone-assisted E3 ubiquitin ligase, ubiquitylates nNOS in cells, as well as in an in vitro system containing purified E1 ubiquitin-activating enzyme, an E2 conjugating enzyme (UbH5a), CHIP, glutathione S-transferase-tagged ubiquitin, and an ATP-generating system (Peng et al., 2004). The addition of purified hsp70 and hsp40 to this in vitro system greatly enhances the amount of nNOS-ubiquitin conjugates, suggesting that CHIP is an E3 ligase for nNOS whose action is facilitated by, and possibly requires, its interaction with nNOS-bound hsp70. This raises the possibility that hsp70 directly mediates protein triage decisions by recognition of destabilized nNOS and recruiting ubiquitin ligase machinery that involves CHIP. It remains to be determined if the guanabenz-treated nNOS is preferentially recognized by the hsp70-based chaperones in this manner. However, we did demonstrate that guanabenz-inactivated nNOS was labilized for ubiquitylation in an in vitro system that contains a crude preparation of reticulocyte proteins, including hsp70.

The molecular mechanism by which dysfunctional forms of nNOS are recognized must be a fundamental biological process that maintains the quality of the nNOS protein in cells. We describe here how guanabenz may perturb this regulatory process to cause a prolonged decrease in nNOS activity and protein levels. The xenobiotic-mediated depletion of tetrahydrobiopterin may be important in understanding how the formation of an SDS-resistant dimer.

References


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