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TETRAHYDROBIOPTERIN PROTECTS AGAINST GUANABENZ-MEDIATED INHIBITION OF NEURONAL NO SYNTHASE IN VITRO AND IN VIVO

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Running title: Mechanism of inhibition of NO synthase by guanabenz

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Abbreviations used: NO, nitric oxide; NOS, nitric oxide-synthase; nNOS, neuronal nitric oxide-synthase; BH_4, (6R)-5,6,7,8-tetrahydro-L-biopterin; BH_2, 7,8-dihydrobiopterin; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; CHIP, C-terminus of Hsc 70 interacting protein.

Abstract

It is established that guanabenz inhibits neuronal 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 ^{14}C-labeled guanabenz, we were unable to detect any guanabenz metabolites or guanabenz-nNOS adducts, indicating that reactive intermediates of guanabenz likely 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.

Nitric oxide 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) and brain cortex (Dambrova et al., 2003) after administration of the drug to rats. Interestingly, the loss of activity is concomitant with the loss of immunodetectable nNOS in penile tissue (Nakatsuka et al., 1998). Consistent with this finding, guanabenz inhibits nNOS and enhances the proteasomal degradation of the enzyme in HEK 293 cells (Noguchi et al., 2000). Guanabenz causes the time- and NADPH- dependent inhibition of nNOS 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 nNOS, such as N^G-methyl-L-arginine and N^5-(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 as reversible inhibitors, such as N^G-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 nNOS renders the enzyme susceptible for degradation. Although guanabenz is well characterized with respect to degradation of nNOS in cells and in rats, relatively little is known about how guanabenz inhibits nNOS. In the current study, we chose to address this question with the use of purified nNOS in the hopes of understanding what dysfunctional forms of nNOS are recognized for degradation. We found that guanabenz causes a tetrahydrobiopterin (BH_4)-deficient state of nNOS due to the oxidative destruction of the pterin that is facilitated by the presence of NADPH. The addition of BH_4

completely reactivates this dysfunctional form of the enzyme. The administration of BH_4 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.

MATERIAL AND METHODS

Materials

Guanabenz was purchased from Research Biochemicals International (Natick, MA). Glucose-6-phosphate, glucose 6-phosphate dehydrogenase, $NADP^+$, N^G-nitro-L-arginine, L-arginine, D-arginine, 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). (6R)-5,6,7,8-tetrahydro-L-biopterin (BH_4) was purchased from Dr. Schirk's Laboratory (Jona, Switzerland). The affinity-purified rabbit IgG against brain NOS used for immunoblotting nNOS was from BD Biosciences Transduction Laboratories (Lexington, KY). [Benzylidene carbon ^{14}C]-labeled guanabenz (56 mCi/mmol) was custom synthesized by Du Pont NEN (Boston, MA). L-[$^{14}C(U)$]-arginine (330.0 mCi/mmol) and ^{125}I-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 previously described (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 $CaCl_2$, 2500 unit/ml superoxide dismutase, 1250 units/ml catalase, 20 µg/ml pure calmodulin, 0.23 mg/ml bovine serum albumin, and an NADPH-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 $CaCl_2$, 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

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oxyhemoglobin was monitored by measuring the absorbance at λ401 nm-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 (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 (Klatt 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 % bromophenol 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 6 % SDS-PAGE. Proteins were then transferred to nitrocellulose membranes (0.2 µm, BioRad) and probed with 0.1 % anti-nNOS. The immunoblots were then incubated a second time with ^{125}I-conjugated goat anti-rabbit IgGs to visualize the immunoreactive bands. The membranes were dried and exposed to X-OMAT film 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 (Jianmongkol 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 x 15 cm) 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 acetonitrile) was run over 30 min and 5 min, respectively. Absorbance at 220 nm and 400 nm was monitored.

Quantification of BH_4 and BH_2 – The amounts of BH_4 and BH_2 in the reaction mixtures were determined by use of an HPLC fluorescence method as described by Klatt et al (Klatt et al., 1996). The method involves oxidization of BH_4 and BH_2 to biopterin by treatment with KI/I_2 solution under acidic conditions. To give the specific amount of BH_2, the KI/I_2 oxidation is done in a basic solution where BH_4 and BH_2 are oxidized to pterin and biopterin, respectively. Specifically for oxidation under acidic conditions, a 40-µl aliquot of the first reaction mixture was treated with 10 mM I_2 and 50 mM KI in a total volume of 50 µL of 100 mM HCl for 1 hr 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 x 250 mm) equilibrated with 20 mM NaH_2PO_4, pH 3, with 5% methanol at a flow rate of 1 ml/min. The pterins 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 pterins was performed with the use of a Waters systems described above and an Applied Biosystems Spectroflow 980 fluorescence detector. To oxidize the pterins 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 ^{14}C-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 22ºC. An aliquot (75 µl) was injected onto a reverse phase HPLC column (C4 Vydac 5 µm, 2.1 x 150 mm) equilibrated with solvent A (0.1% TFA) at a flow rate of 0.3 ml/min. A linear gradient to 75% solvent B (0.1% TFA in acetonitrile) 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, Downers Grove, IL)

Treatment of animals, sample preparation, and activity assays – Guanabenz was dissolved in physiological saline and administrated to male Wistar rats (150-250 g) at the indicated doses by

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intraperitoneal injection at 9:00 A.M. and 6:00 P.M. BH_4 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 1-2 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 DTT, 10 μg/ml trypsin inhibitor, 10 mg/ml of leupeptin, 2 μg/ml of aprotinin, 1 mg/ml phenylmethanesulphonyl fluoride, and 100 μM BH_4) with the use of a metal tissue mincer (SDT Tissumizer®, Tekmar, Cincinnati, OH). The homogenate was centrifuged at 245,000 x g 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, Hercules, CA) 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 $CaCl_2$, 1 mM NADPH, 30 μM [^{14}C]-arginine (60 mCi/mmol), 100 μM BH_4, 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 [^{14}C]-citrulline was determined as previously described (Nakatsuka et al., 1998). The formation of [^{14}C]-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 M column (PD-10, Pharmacia Biotech, Piscataway, NJ) preequilibrated in 10 mM Hepes, pH 7.5, containing 320 mM sucrose, 100 μM EDTA, 1.5 mM DTT, 10 μg/ml trypsin inhibitor, 10 mg/ml of leupeptin, 2 μg/ml of aprotinin, and 1 mg/ml phenylmethanesulphonyl fluoride to remove endogenous arginine and excess BH_4. An aliquot (1.2 mg) of the gel-filtered fraction was placed in a 'reaction mixture' containing 1 mM $CaCl_2$, 1 mM NADPH, 10 μg/ml calmodulin, and the desired concentration of guanabenz, in a total volume of 1 ml of 40 mM potassium phosphate, pH 7.4. Aliquots (150 μl) were taken

from the reaction mixture and placed in the citrulline assay mixture and the activity was determined as described above.

SDS PAGE and Western blotting – The penile supernatant fraction (15 µg of protein) was analyzed with the use of SDS-PAGE (4-12% gradient gel) as previously described (Nakatsuka et al., 1998). The gels were blotted onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH), blocked with 0.2 mg/ml thimerosal in Blotto solution (Advanced Biotechnologies Inc., Columbia, MD), and probed (1:250) with a mouse monoclonal antibody against brain NOS (Transduction Laboratories, Lexington, KY). An anti-mouse IgG antibody (1:10,000) conjugated to peroxidase (Boehringer Mannheim, Indianapolis, IN) was used as a secondary antibody. An ECL reagent (Amersham Life Science Inc., Arlington Heights, IL) and X-OMAT film (Kodak, Rochester, NY) was 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 insure that the density was linearly dependent on the amount of NOS over the relevant concentration range.

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 specific detection of nNOS-ubiquitin conjugates has also been established (Kamada et al., 2005). Fraction II was prepared from rabbit reticulocyte lysates as previously described (Hershko et al., 1983). 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, 15 µM ubiquitin, an ATP-regenerating system (2 mM ATP, 10 mM creatine phosphate, 5 mM $MgCl_2$, and 10 units/ml creatine phosphokinase), and 0.4 mg/ml of fraction II. An aliquot (20 µl) of the samples were quenched with 20 µl of sample buffer containing 5% SDS, 20% glycerol, 100 mM dithiothreitol, and 0.02% bromophenol 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 x 8 cm). Proteins were then transferred to nitrocellulose membranes (0.2 μm, BioRad) and probed with 0.5% anti-ubiquitin (DAKO, Carpinteria, CA). The immunoblots were then incubated a second time with ^{125}I-conjugated goat anti-rabbit IgGs to visualize the immunoreactive bands. The membranes were dried and exposed to X-OMAT film for 1 h at -80°C. The bands corresponding to nNOS-ubiquitin were excised and the radioactivity quantified by the use of a gamma counter.

Statistical analysis – All values are reported as the mean ± standard error (S.E.). An upaired *t* test was used to compare values. Statistical significance was considered 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.

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Results

Guanabenz-mediated inhibition of purified nNOS — It is established that guanabenz inhibits nNOS and enhances the proteolytic turnover of nNOS protein in cells (Noguchi et al., 2000). Consistent with this, administration of guanabenz to rats decreases nNOS activity and protein (Nakatsuka et al., 1998; Dambrova et al., 2003). Moreover, the time-dependent inhibition of nNOS 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 nNOS 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 min 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 nNOS 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 nNOS 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 nNOS that is preferentially ubiquitylated and proteasomally degraded (Noguchi et al., 2000). Recently, it was found that destabilization of the dimeric functional form of nNOS correlates with recognition for proteasomal degradation (Dunbar et al., 2004). Thus, we asked if guanabenz destabilizes nNOS 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 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*), likely 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- and concentration- dependence of the loss of dimeric nNOS reflects the loss of nNOS activity seen above. These results are entirely consistent with the notion that destabilization of the dimer by guanabenz generates some altered nNOS 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 one-half of 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

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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 BH_4 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 BH_4 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 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 (*c.f. 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 (*c.f. 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 likely 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 (*c.f. solid triangles* with *open triangles*). Also, 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 due to N^G-nitro-L-arginine, a slowly reversible active site directed inhibitor (*X*). It is noteworthy that N^G-nitro-L-arginine stabilizes the SDS-resistant dimeric form of nNOS (Dunbar et al., 2004). Thus, guanabenz

appears 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 dihydrobiopterin (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 μM BH_4 is sufficient for complete reversal, consistent with the approximate loss of BH_4. The restoration of activity is rapid, as 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 of nNOS in the presence of NADPH (Fig. 4C). The addition of dihydropteridine reductase, which reduces BH_2 to BH_4, abolishes the inhibition of nNOS due to guanabenz (Fig. 4C), indicating that the oxidation of BH_4 to BH_2 and the subsequent formation of a BH_4-deficient nNOS is the cause of the nNOS inhibition. Consistent with this finding, BH_4 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, BH_4 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 1,000 units/ml (*solid circles*). Catalase at

concentrations up to 400 units/ml has no effect (*solid triangles*). In a previous study, 100 units/ml of 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 appears 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 BH_4 (Fig. 5B).

Studies with radiolabeled guanabenz — The purified nNOS was treated with radiolabeled guanabenz to determine if 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 (CAM) 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 BH_4 deficiency is the major mechanism of nNOS inhibition.

Tetrahydrobiopterin protects against guanabenz-mediated inhibition of penile NOS activity in rats — We sought to determine if the inhibition and loss of nNOS protein *in vivo* could be ameliorated by BH_4. We utilized a previously established procedure for treatment of rats with 5 mg/kg/day guanabenz for four 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 previously described (Nakatsuka et al., 1998). The concurrent administration of 200 mg/kg/day of BH_4 completely abrogates the inhibitory effect of guanabenz and prevents the loss of nNOS protein. The administration of the same dose of BH_4 alone has no effect on NOS activity or level of nNOS protein. The dose dependence of the protection by BH_4 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 BH_4 on nNOS are not as well characterized and we know of no studies in penile tissue. To further examine the role of BH_4 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 decreases nNOS activity by approximately one-half after treatment for 15 min (*solid circles*). We found that the addition of BH_4 in the reaction mixture completely prevents the inactivation due to guanabenz, with a concentration of 1µM giving nearly complete protection. The concentration dependence of BH_4 found here is highly similar to the concentration dependence reported for the activation of nNOS in desalted rat cerebellar cytosol where 1 µM BH_4 was required for maximal activation of nNOS (Nunokawa et al., 1992). The addition of BH_4 does not increase the activity of the untreated sample (*X*) nor protect against the inhibition by N^G-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 upper 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, lower 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 proteasomal 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. In order 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 due to the guanabenz-mediated, nNOS-catalyzed destruction of BH_4, which stabilizes the active dimeric state of nNOS. Moreover, the treatment of rats with BH_4 completely protects from the guanabenz-mediated inhibition and loss of penile nNOS. Although this suggests that the loss of BH_4 is a mechanism for the inhibition and loss of nNOS *in vivo*, further studies on BH_4 are needed to fully understand the molecular mechanisms responsible.

Superoxide dismutase, but not catalase, prevents the loss of BH_4 from purified nNOS treated with guanabenz, indicating that nNOS-derived superoxide is responsible for the loss of BH_4. The reaction of 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 appears 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 as 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 N^G-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 (UbcH5a), CHIP, GST-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 redox regulation of BH_4 may be important in understanding how chemicals inhibit and cause the loss of nNOS *in vivo*. The

interactions of drug molecules in protein quality control will likely be an important pharmacological and toxicological consideration in the development of safer and more effective drugs.

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Footnotes

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Figure legends

Fig. 1**. Guanabenz-mediated inactivation of purified nNOS.** *A,* indicates the time-dependent loss of nNOS activity due to 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. *denotes NOS activity was significantly ($p < 0.05$) lower for the guanabenz treated sample than that for control. *B,* indicates the effect of varying the concentration of guanabenz in the first reaction mixture. The amount of guanbenz 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. *denotes significantly ($p < 0.05$) different from untreated. The values are the mean ± S.E. (n = 3).

Fig. 2. **Effect of guanabenz on the amount of SDS-resistant dimer of nNOS**. The nNOS was treated with guanabenz as described 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 ^{125}I-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. *denotes significantly ($p < 0.05$) lower dimer for guanabenz-treated versus untreated. #denotes 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. *denotes significantly ($p < 0.05$) lower dimer than untreated.

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#denotes significantly ($p < 0.05$) higher monomer than untreated. The values are the mean ± S.E. (n = 3).

Fig. 3. **Effect of substrate, NADPH, tetrahydrobiopterin, and calmodulin, on the guanabenz-mediated inactivation of nNOS**. *A*, indicates 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 μM guanabenz (*open bars*) or untreated (*solid bars*). *B*, indicates the protective effect of BH_4 on the inhibition of nNOS caused by guanabenz when superoxide dismutase and catalase were omitted from the first reaction mixture described in *Methods*. nNOS was either treated with 100 μM guanabenz (*circles*) or untreated (*squares*). Open symbols, NADPH was omitted from the first reaction mixture; closed symbols, NADPH was present in the first reaction mixture. *C*, indicates the protective effect of BH_4 on the extent of inhibition of purified nNOS caused by 100 μM guanabenz (*closed triangles*), 10 μM N^G-nitro-L-arginine (*X*), or untreated (*open triangles*) in the first reaction mixture. This mixture contained superoxide dismutase, catalase, and NADPH. The values are the mean ± S.E. (n = 3). *denotes significantly ($p < 0.05$) lower activity for treated versus untreated.

Fig. 4. **Guanabenz causes the loss of nNOS activity and tetrahydrobiopterin**. *A*, guanabenz causes the loss of BH_4. The nNOS was treated with 100 μM guanabenz for 20 min in the first reaction mixture as indicated in *Methods*, except that NADPH was omitted. The amount of BH_4 present in the first reaction mixture was measured by HPLC and compared to the nNOS activity. The presence of calmodulin and guanabenz are as indicated. *B*, the activity loss due to guanabenz is reversed by addition of BH_4 to the oxyhemoglobin assay mixture. The activity was measured by the oxyhemoglobin assay containing the indicated amounts of BH_4. Closed squares, nNOS treated with guanabenz; open squares, untreated nNOS. *C*, the conditions were as in A, except that NADPH was present in the first reaction mixture. All samples contained calmodulin and some samples contained 10 units/ml of dihydropteridine reductase (DHPR) as

indicated. *D*, the conditions were as in B, except that NADPH was present in the first reaction mixture. The values are the mean ± S.E. (n = 3). *denotes significantly ($p < 0.05$) lower values for guanabenz treated versus untreated.

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 in *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), 2,500 units/ml superoxide disumutase (SOD), or 1mM NADPH was omitted from the reaction mixtures as indicated. The values are the mean ± S.E. (n = 3). *denotes significantly ($p < 0.05$) lower values relative to untreated.

Fig. 6**. HPLC profile of nNOS treated with ^{14}C-labeled guanabenz in the absence or presence of calmodulin.** The nNOS was treated with radiolabeled guanabenz as described in *Methods. A*, indicates nNOS treated with guanabenz in the absence of calmodulin. *B*, indicates nNOS treated with guanabenz in the presence of calmodulin. The residual activity was 85% and 38% for the untreated and guanabenz treated sample, respectively. G, guanabenz; NOS, nNOS; CAM, calmodulin.

Fig. 7**. Tetrahydrobiopterin protects against guanabenz-mediated inactivation and loss of penile nNOS in rats.** *A*, indicates the effect of guanabenz (5mg/kg/day) and BH_4 (200

mg/kg/day) on penile NOS activity (*solid bars*) and penile nNOS protein (*open bars*) after treatment of rats for four days. The treatment of the rats and the measurement of activity by the citrulline assay are as described in *Methods.* *denotes significantly ($p < 0.05$) lower values relative to untreated. *B,* indicates the dose response of BH_4 on the penile nNOS activity under conditions of *A.* *denotes significantly ($p < 0.05$) higher activity relative to untreated. *C,* indicates the treatment of penile cytosol *in vitro* with guanabenz and BH_4. The inactivation of nNOS activity was determined with the use of the reaction mixture and citrulline assay mixture as described in *Methods.* The reaction mixture was incubated for 15 min in the presence of the following: closed circles, 100 μM guanabenz; open circles, 5 μM N^G-nitro-L-arginine; X, untreated. The indicated amounts of BH_4 were added to the reaction mixture. The values are the mean ± S.E. (n = 3). *denotes significantly ($p < 0.05$) lower activity relative to untreated.

Fig. 8**. Guanabenz-treated nNOS is labilized for ubiquitylation in an in vitro system containing fraction II.** Purified nNOS was treated with 100 μM guanabenz (G) and then placed in a reaction mixture containing ubiquitin, ATP, and fraction II. The generation and detection of the nNOS ubiquitin conjugates are as described in *Methods*. The values are the mean ± S.E. (n = 3). *denotes significantly ($p < 0.05$) higher nNOS-Ub conjugates relative to untreated.

Figure 1

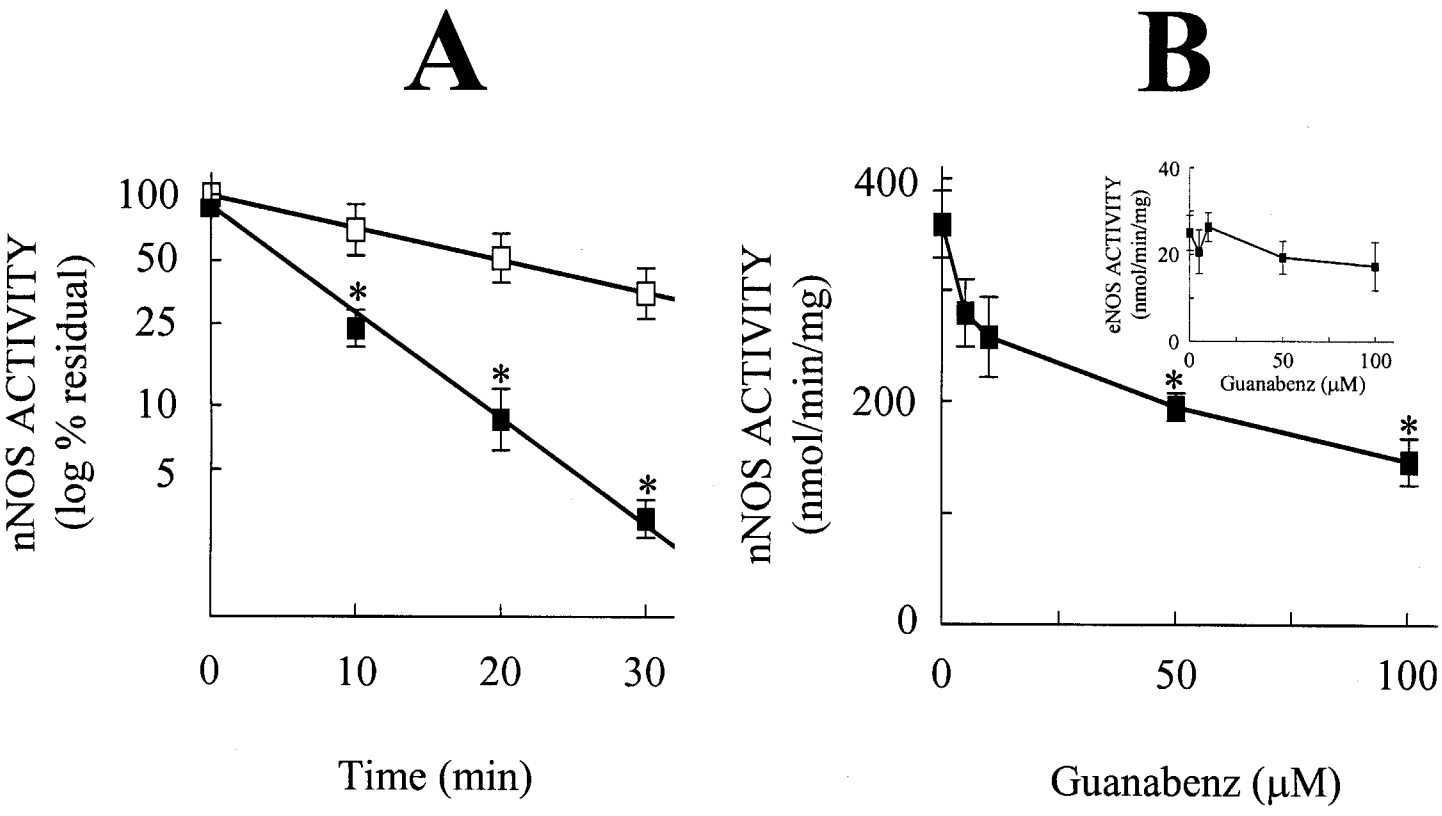

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Figure 2

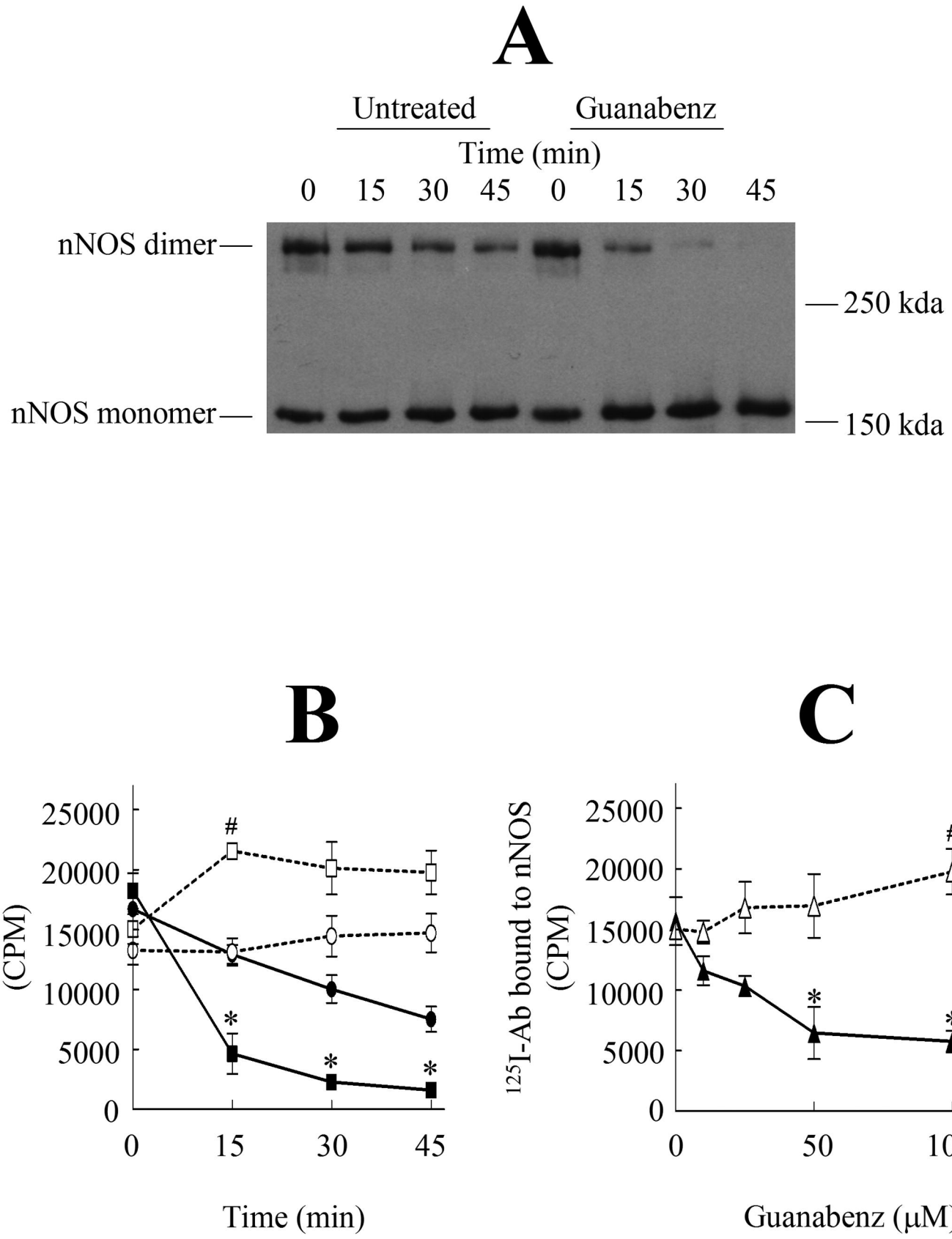
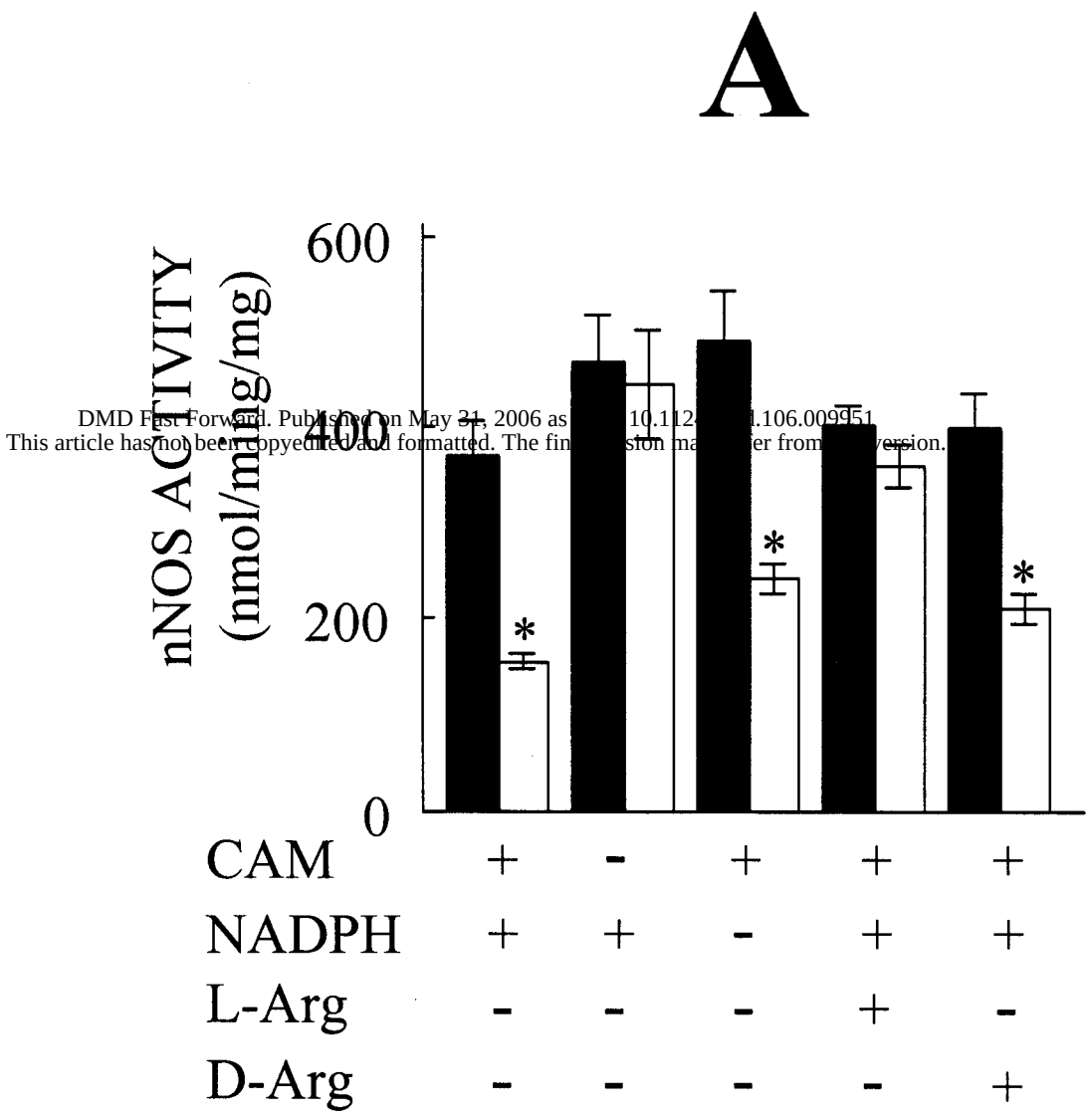


Figure 3



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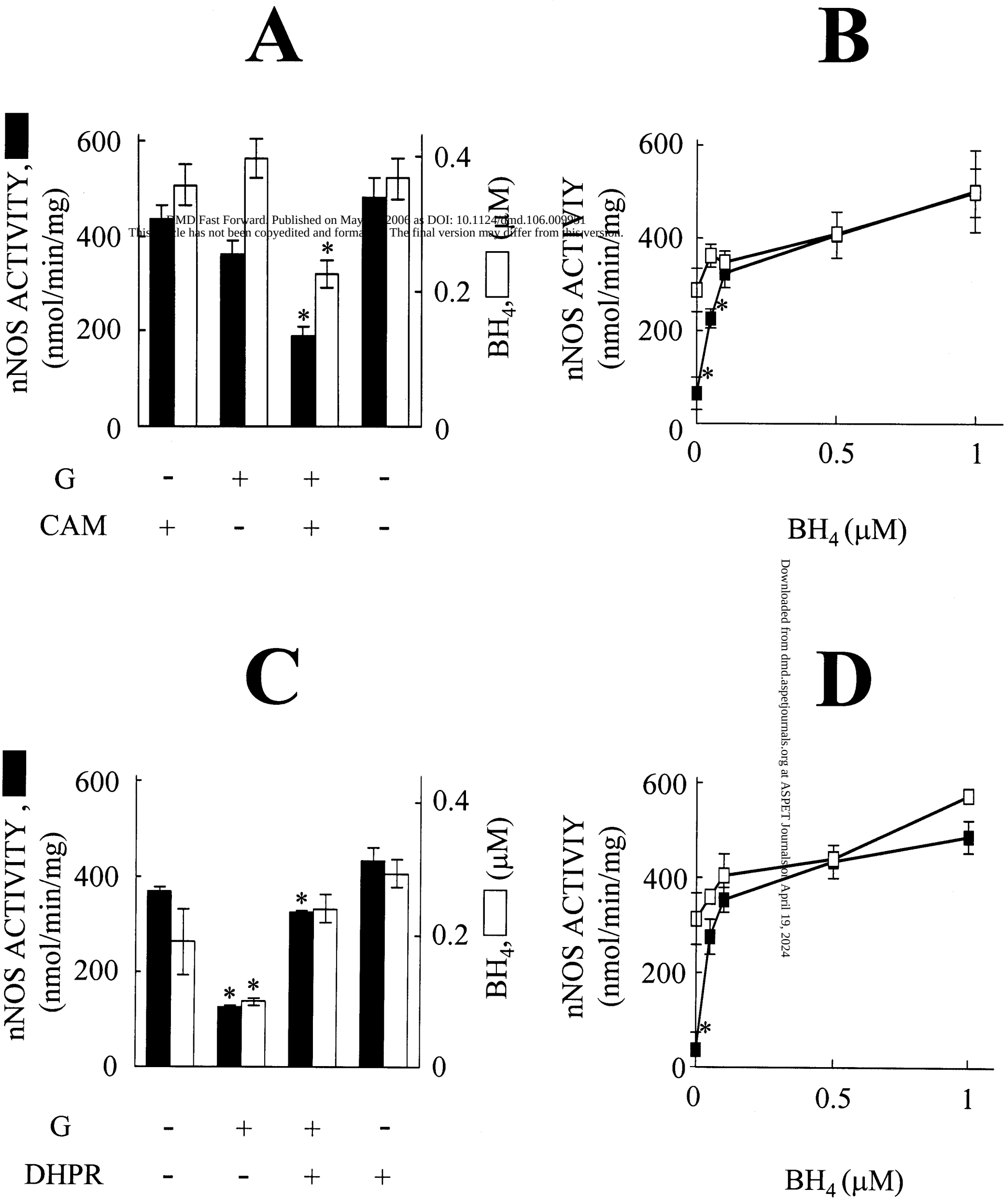

Figure 5

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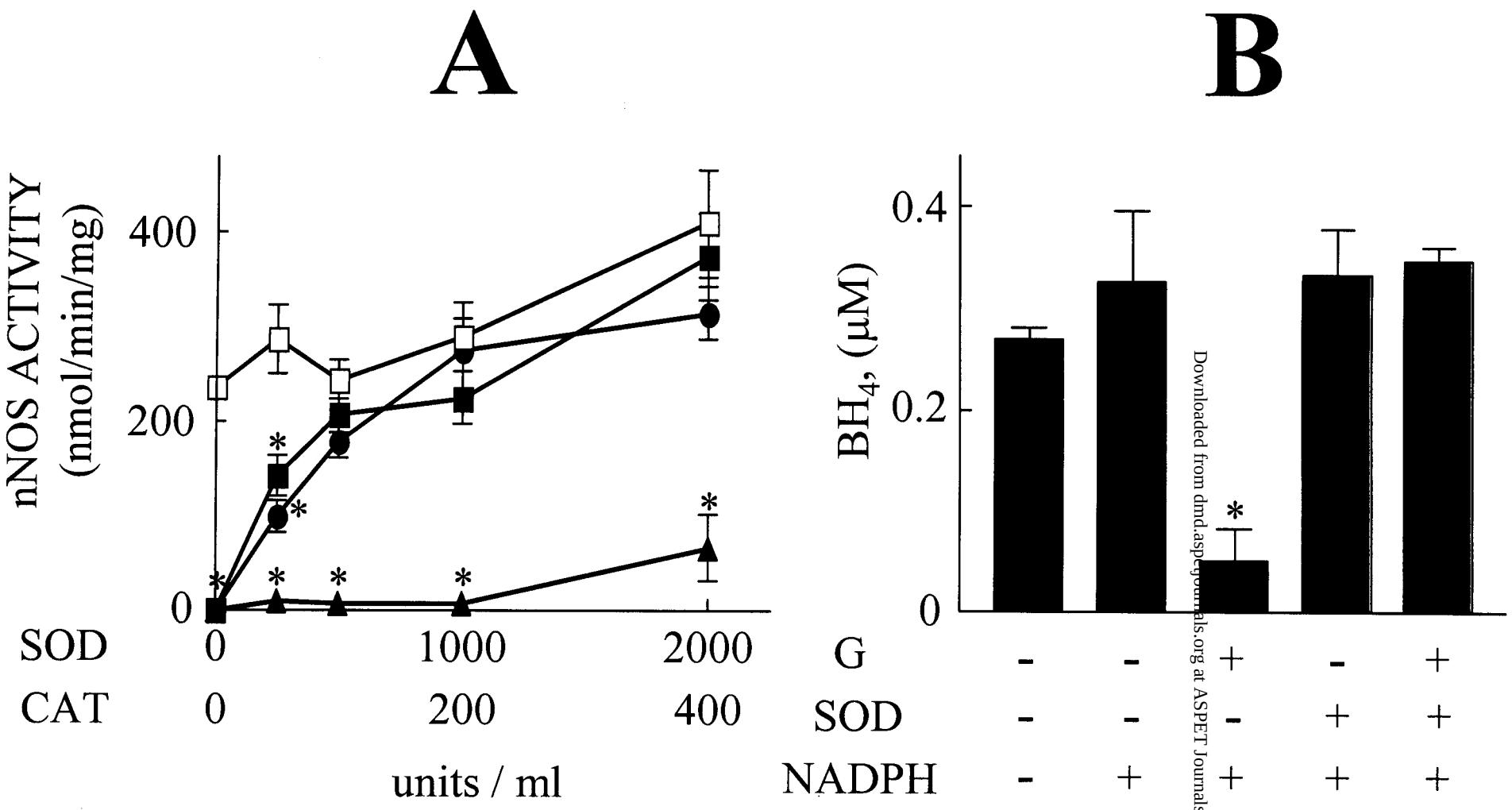

Figure 6

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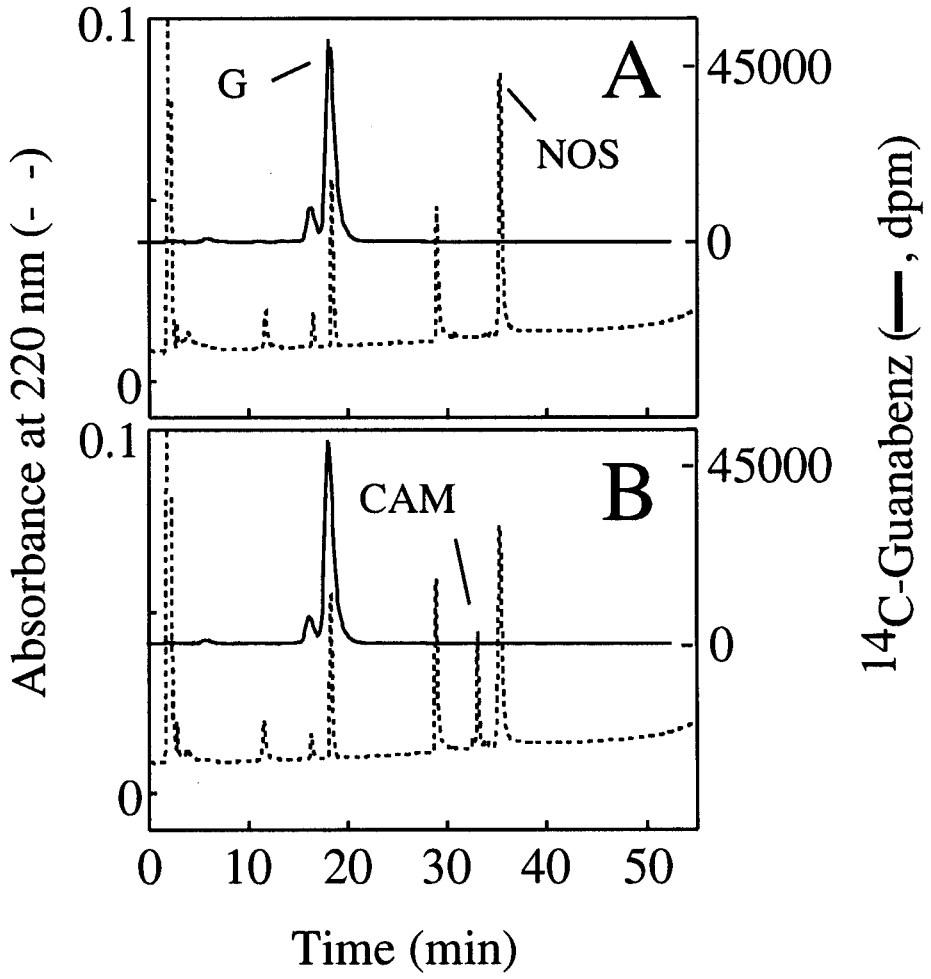
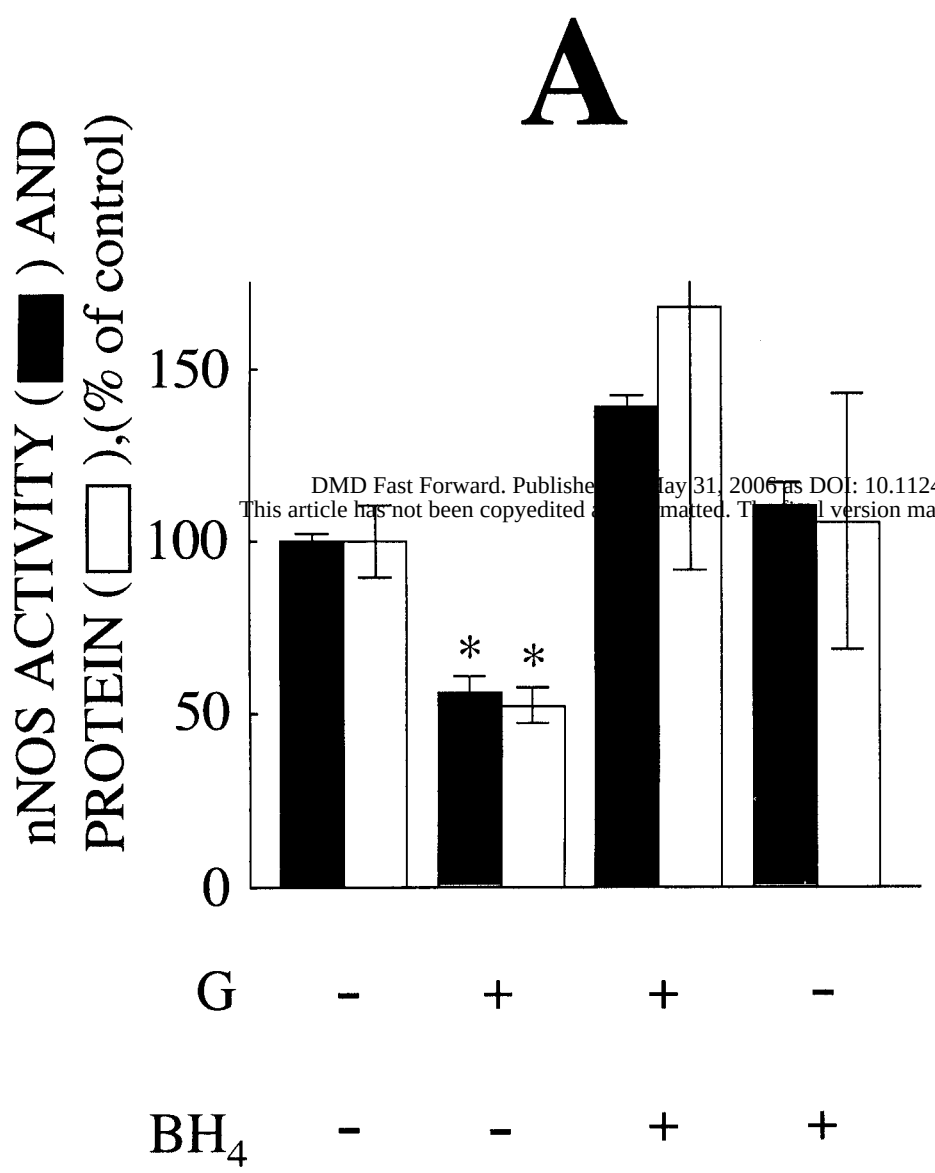
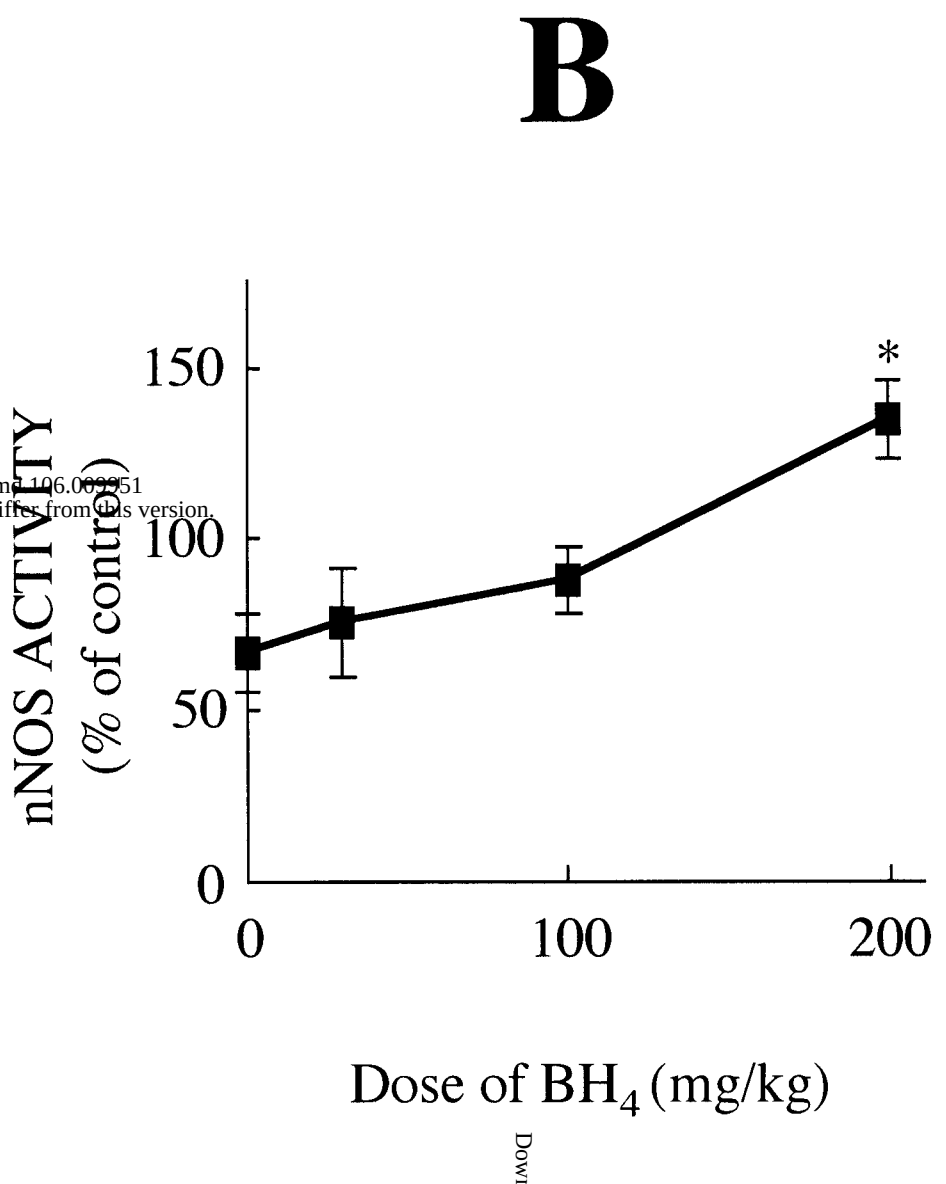


Figure 7





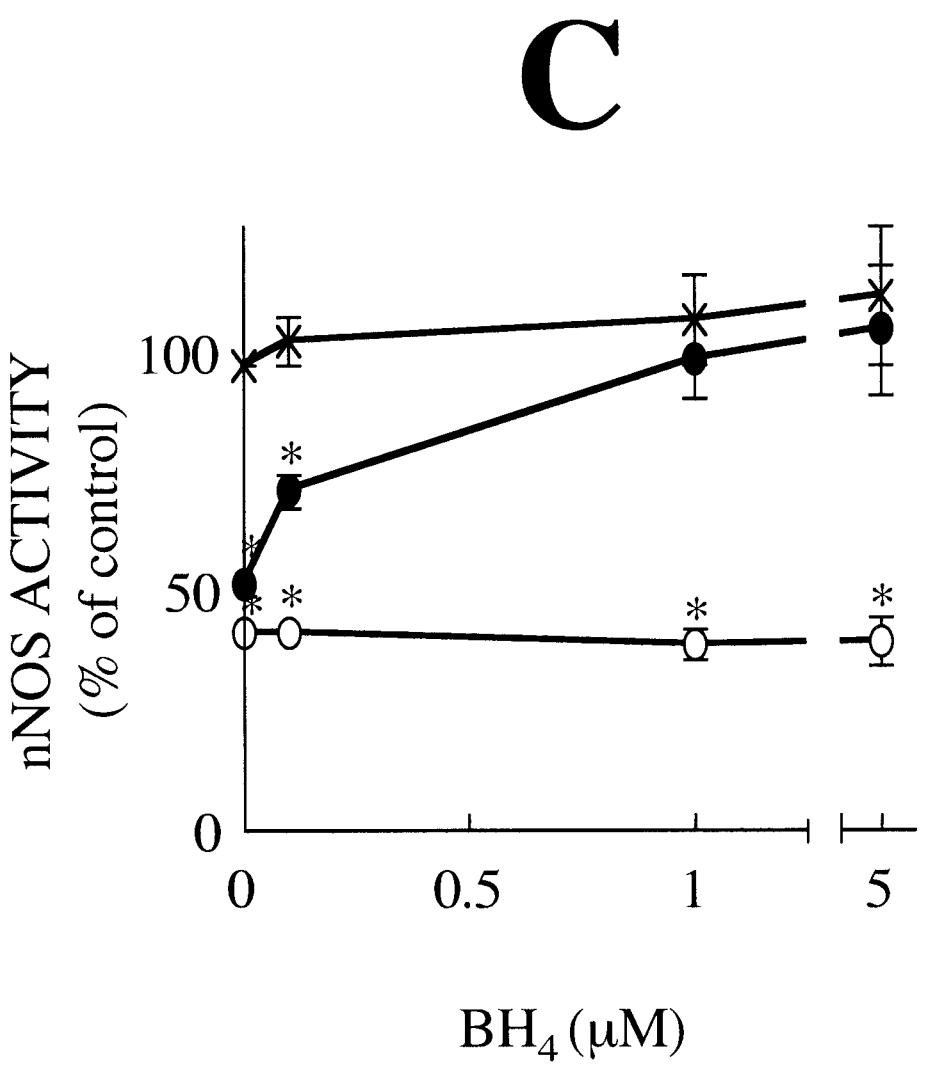


Figure 8