METABOLISM-BASED INACTIVATION OF PENILE NITRIC OXIDE SYNTHASE ACTIVITY BY GUANABENZ

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(Received September 30, 1997; accepted January 28, 1998)

This paper is available online at http://www.dmd.org

ABSTRACT:

Guanabenz (Wytensin) was shown to inactivate nitric oxide synthase (NOS) activity in vitro and in vivo. In in vitro studies with the use of a cytosolic fraction from penile tissue, the inactivation was found to depend on NADPH, time, and the concentration of guanabenz. The L-, but not the D-, isomer of arginine could protect from the inactivation, suggesting an active site-directed event. The kinetics of inactivation could be described by an apparent dissociation constant for the initial reversible complex (K_i) and a pseudo first-order inactivation constant (k_inact) of 38.5 μM and 0.179 min^-1, respectively. In in vivo studies, guanabenz was shown to inhibit penile cytosolic NOS activity in a dose- and time-dependent manner. Treatment of rats with guanabenz (5 mg/kg/day) for 4 days caused a decrease of approximately one-half in the NOS activity of the penile cytosolic fraction with a concomitant loss in the amount of immunodetectable NOS protein. Treatment for 4 days at a dose of 0.5 mg/kg/day showed a similar decrease in activity, whereas a dose of 0.05 mg/kg/day showed no effects. Due to the multitude of processes that are regulated by NO, the inactivation of NOS is a potential mechanism to be considered in a variety of biological effects associated with drugs.

Nitric oxide, the radical metabolite formed from the metabolism of L-arginine by nitric oxide synthase (NOS), has been shown to be involved in a variety of physiological processes, including neurotransmission, vasorelaxation, platelet aggregation, and penile erection as well as in a variety of pathological conditions including septic shock, reperfusion injury, arthritis, atherosclerosis, diabetes, and graft rejection (Burnett et al., 1992; Forstermann et al., 1994; Moncada et al., 1991; Schmidt and Walter, 1994). NOS was recently shown to be a hemoprotein of the cytochrome P450 type (McMillan et al., 1992; Stuehr and Ikeda-Saito, 1992; White and Marletta, 1992). In accord with this finding, suicide inactivators of liver P450 cytochromes, such as phenychlidine (Hoag et al., 1984; Osawa and Coon, 1989; Osawa and Pohl, 1989), and CBrCl3 (Osawa and Pohl, 1989), are suicide inactivators of NOS (Osawa and Davila, 1993; Osawa et al., 1994). Suicide or metabolism-based inactivators of liver microsomal P450 cytochromes have been important tools for probing the mechanism of catalysis, identifying active site residues, and for assessing the biological roles of P450 metabolites as well as for studying their turnover (Halpert et al., 1994). Suicide inactivators of NOS may provide similar insights.

In the course of such studies, we discovered that guanabenz (Wytensin, for structure see fig. 1), a clinically used antihypertensive agent, caused the inactivation of cytosolic penile NOS in vitro and in vivo. The current paper describes our findings and characterization of the kinetics of this inactivation process. The inactivation caused by guanabenz in vivo was comparable with that caused by N⁴-nitro-L-arginine, a known inhibitor of NOS (Dwyer et al., 1991). Guanabenz may be a useful tool for future studies on the mechanisms of inactivation of NOS. In addition, we are not aware of any articles describing the inactivation of liver microsomal P450 cytochromes by guanabenz, in contrast to that for phenychlidine or CBrCl3, described above. Moreover, as NO plays a key role in a variety of physiological processes, the inactivation of NOS by xenobiotics, such as drugs, is of potential biomedical importance.

Materials and Methods

Materials. Guanabenz was purchased from Research Biochemicals International (Natick, MA). N⁴-Nitro-L-arginine, L-arginine, D-arginine, and NADPH were purchased from Sigma. Male Wistar rats were purchased from Charles River Laboratories (Wilmington, MA). (6R)-5,6,7,8-tetrahydro-L-biopterin (tetrahydrobiopterin) was purchased from Dr. Schirks Laboratory (Jona, Switzerland). L-[¹⁴C(U)]arginine (330.0 mCi/mmol) was purchased from Du Pont NEN.

Treatment of Animals and Sample Preparation. Guanabenz or N⁴-nitro-L-arginine was dissolved in physiological saline and administered to male Wistar rats (150–250 g) at the indicated doses by intraperitoneal injection at 9:00 a.m. and 6:00 p.m. Rats were sacrificed by decapitation 16 hr 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 dithiothreitol, 10 μg/ml trypsin inhibitor, 10 mg/ml leupeptin, 2 μg/ml aprotinin, 1 mg/ml phenylmethylsulfonyl fluoride, and 100 μM tetrahydrobiopterin (Knowles et al., 1990)] with the use of a metal tissue micer (SDT Tissumizer, 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 concentrations of these samples were determined by the method of Bradford (Bio-Rad) with the use of bovine serum albumin as a standard. Rat brain cytosol was prepared with the use of the same buffers as for penile tissue as described previously (Knowles et al., 1990).

**Enzyme Assays.** The NOS activity of samples from the *in vivo* studies were determined by adding the supernatant fraction (0.6 mg) to an “assay mixture” containing 1 mM CaCl₂, 1 mM NADPH, 30 μM [¹⁴C]arginine (60 mCi/mmol), 100 μM tetrahydrobiopterin, 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 previously described (Oswa et al., 1994). The formation of [¹⁴C]citrulline was linear over the 10-min period. In control experiments, supernatant fractions (0.6 mg) from untreated and treated rats were mixed into the assay mixture to show that the inhibition of NOS was not due to changes in endogenous arginine levels or to the formation of an inhibitory metabolite. For experiments on the inactivation of NOS *in vitro*, the supernatant fraction from untreated rats was loaded onto a Sephadex G-25 M column (PD-10, Pharmacia Biotech Inc.) preequilibrated 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 phenylmethanesulfonyl fluoride to remove endogenous arginine and excess tetrahydrobiopterin. An aliquot (1.2 mg) of the gel-filtered fraction was placed in a “first reaction mixture” containing 1 mM CaCl₂, 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 first reaction mixture and placed in the assay mixture, and the activity was determined as described above.

**Western Blot.** The penile supernatant fraction (15 μg of protein) was analyzed with the use of SDS-polyacrylamide gel electrophoresis (4–12% gradient gel). The gels were blotted onto a nitrocellulose membrane (Schleicher & Schuell), 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) was used as a secondary antibody. An ECL reagent (Amersham) 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). 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**

**Inactivation of NOS Activity by Guanabenz *in Vitro.*** The presence of guanabenz in the first reaction mixture containing the supernatant fraction prepared from penile tissue caused the time-dependent inactivation of NOS activity (fig. 2A). This inactivation was dependent on the concentration of guanabenz, and the loss of activity seemed to follow pseudo first-order kinetics with an apparent dissociation constant for the initial reversible complex (Kᵢ) and an inactivation constant (kᵢₐct) of 38.5 μM and 0.179 min⁻¹, respectively (fig. 2B). A complete dependence on the presence of NADPH in the first reaction mixture was observed for the inactivation process (fig. 2C).

**Discussion**

Guanabenz was shown to inactivate NOS activity in the cytosolic fraction of brain and penile tissue by a process that required NADPH,
The inactivation of NOS was determined with the use of a “first reaction mixture” and an “assay mixture” as described in Materials and Methods. (A) Extent of inhibition of NOS activity at the following concentrations of guanabenz in the first reaction mixture: □, untreated; ○, 5 µM; ●, 10 µM; △, 20 µM; □, 50 µM; ●, 100 µM. (B) Replot of the data from A. (C) Extent of inhibition of NOS with guanabenz (30 µM) in the absence of NADPH (○) or in the presence of NADPH (●) in the first reaction mixture. A control without guanabenz and NADPH is also shown (□). (D) Extent of inhibition of NOS with guanabenz (30 µM, □) and the effect of L-arginine (30 µM, ○) or D-arginine (30 µM, ●) in the first reaction mixture.

The brain cytosolic fraction (7.2 mg/ml) was placed in a reaction mixture containing guanabenz (100 µM) and NADPH (0.1 mM) in 0.6 ml of 40 mM potassium phosphate, pH 7.4, similar to that described previously (Osawa and Davila, 1993). The mixture was incubated at room temperature for 1 hr. The mixture was then placed onto a Sephadex G-25 column as described in Materials and Methods. The NOS activity was determined by the oxyhemoglobin method as previously described (Osawa and Davila, 1993). The values are the mean and standard deviation (N = 3).

The inactivation of NOS was dependent on time as well as the concentration of guanabenz, specifically protected the enzyme from the inactivation. The inactivation seemed to be an active site-directed event as the natural substrate of NOS stereospecifically protected the enzyme from the inactivation. The inactivation of NOS, including several derivatives of arginine. For example, the N⁶-methyl, N⁷-allyl, and N⁷-amino analogs of arginine inactivate NOS isolated from stimulated macrophages with apparent Kᵢ values of 4.2 µM and 0.05 min⁻¹, 3.4 µM and 0.026 min⁻¹, and 3 µM and 0.26 min⁻¹, respectively (Olken et al., 1991; Olken and Marletta, 1992; Wolff and Lupeski, 1996). The N⁷-methyl-L-arginine is also a metabolism-based inactivator of the neuronal NOS with apparent Kᵢ and kᵢnact values of 2 µM and 0.022 min⁻¹, respectively (Feldman et al., 1993; Reif and McCready, 1995). Other guanidine-containing compounds, such as aminoguanidine (Wolff and Lupeski, 1995) and diaminoguanidine (Wolff and Lupeski, 1996), have been shown to be metabolism-based inactivators of neuronal, macrophage, and endothelial isoforms of NOS. However, methylguanidine and 1,1'-dimethylguanidine are not metabolism-based inactivators (Wolff and Lupeski, 1996). Based on this evidence, it was suggested that the hydrazine moiety was critical for inactivation. The hydrazine moiety on guanabenz may also suffice in conferring the ability to be a metabolism-based inactivator. The exact mechanism of inactivation is currently not known for any metabolism-based inactivator of NOS. Studies on the suicide inactivation of liver microsomal P450 cytochromes (Correa et al., 1981; Halpert et al., 1994; Osawa and Pohl, 1989) and recent studies with NOS (Gerber and Ortiz de Montellano, 1995; Olken et al., 1994) suggest that covalent alteration of the heme prosthetic group and/or the protein are likely mechanisms.

Unlike the other guanidine-containing compounds that were studied, guanabenz is already clinically used. Although the inactivation of...
NOS is not the pharmacological basis of the antihypertensive action of guanabenz, the drug is known to cause impotence as a side effect (Brock and Lue, 1993; Weiss, 1991). This may be of relevance as NO has been shown to play a key role in penile erection (Burnett et al., 1992; Bush et al., 1992; Rajfer et al., 1992) and that inhibitors to NOS can cause impotence (Burnett et al., 1992). In the current study, guanabenz was found to inactivate penile NOS activity in vivo. The NOS activity of the penile cytosolic fraction from rats treated with guanabenz (5 mg/kg/day) for 4 days was approximately one-half of that of the control animals, a level that is comparable with that seen with N\textsuperscript{G}-nitro-L-arginine (5 mg/kg/day) (Dwyer et al., 1991). The nitro compound was chosen as a positive control as previous studies indicate irreversible inactivation of rat brain activity in vivo (Dwyer et al., 1991), although more recent in vitro evidence indicates that the inhibition is slowly reversible (Klatt et al., 1994). The lack of complete inhibition of NOS by N\textsuperscript{G}-nitro-L-arginine at this dose is consistent with that found for the rat brain activity (Dwyer et al., 1991; Iadecola et al., 1994). The inhibition of penile NOS activity by guanabenz after a 4-day treatment was dose dependent with comparable inhibition at 0.5 mg/kg/day. This dose is near the recommended pharmacological dose (0.06 to 0.45 mg/kg/day) for use as an antihypertensive agent in man. Because the inhibition of NOS is time dependent, it is possible that treatment of rats for a period greater than 4 days may decrease the dose needed for observing the inhibition of NOS and may more closely reflect the conditions for use in man. Further work on the physiological effects of guanabenz on penile erection are necessary to determine the significance of these effects. In addition, effects on other isoforms of NOS are warranted, as studies on mice with targeted deletion of the neuronal isoform indicate that the endothelial form can play a prominent role in penile erection (Burnett et al., 1996). It is noteworthy that higher levels of the endothelial isoform were found in the knockout mice in comparison with that of the wild type, perhaps indicating a compensatory mechanism (Burnett et al., 1996). Recently, it has been shown that penile tissue contains an alternatively spliced variant of the brain NOS (Magee et al., 1996), but the impact of these differences on the activity or susceptibility to inactivators of the NOS enzyme has also not been determined. Nonetheless, the current studies clearly indicate that inhibition of NOS by guanabenz can occur in vivo.

The activity loss in vivo was concomitant with a decrease of approximately 50% in the level of immunodetectable NOS protein in the cytosolic fraction prepared from the penis, as visualized by Western blot with the use of a monoclonal antibody that recognizes the amino-terminal portion of neuronal NOS, which is conserved in the penile form. There were no detectable losses in the amount of immunodetectable NOS protein after inhibition with N\textsuperscript{G}-nitro-L-arginine. Thus, the inhibition per se seems not to be the cause for the decrease in steady state levels, in analogy to that found for the liver P450 cytochromes (Bornheim et al., 1987; Correia et al., 1987; Correia et al., 1992; Lunetta et al., 1989; Tierney et al., 1992). We are interested in determining if the metabolism-based inactivation of NOS involves covalent alteration of the protein and subsequent affects on turnover, as has been indicated by studies with liver microsomal P450 cytochromes (Correia et al., 1987; Noguchi et al., 1982; Tierney et al., 1992). Moreover, it would be of interest to determine if long-term treatment with guanabenz leads to a greater loss of NOS or if compensatory mechanisms give rise to induction of the protein.

We report here that drug-induced inactivation of NOS may be a mechanism for toxicological side effects of drugs. Because NO plays a key role in a multitude of important physiological processes, including sexual function, such interactions may explain a variety of toxic effects associated with many drugs. Moreover, the biochemical mechanisms by which this occurs may lead to insight on the regulation of NOS by chemicals, including drugs and other xenobiotics.

Acknowledgments. This work was supported in part by National Institutes of Health Grant ES08365, a grant from the EHS Center in Toxicology at Wayne State, and a PhRMA Foundation Research Starter Grant. Y.O. is grateful for Starting Faculty Awards from the University of Michigan Office of the Vice President of Research, Michigan Memorial Phoenix Project, and the Rackham School of Graduate Studies. Y.O. is a recipient of the Burroughs Wellcome New Investigator Award in Toxicology.

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