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 ($K_{inact}$) 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.

Materials and Methods
Guanabenz was purchased from Research Biochemicals International (Natick, MA). N$\text{N}^3$-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.

The homogenate was centrifuged at 245,000 g for 10 min at 4°C. The homogenate was centrifuged at 245,000 g for 10 min at 4°C.

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2 Abbreviation used is: NOS, nitric oxide synthase.
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 was 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 (Osawa 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 activity present in brain cytosol of the treated rats (fig. 3). The presence of L-arginine, the natural substrate for NOS, protected from this inactivation. The gel filtration of the inactivated supernatant fraction of brain and penile tissue by a process that required NADPH, calmodulin, FMN, and FAD in the assay mixture did not restore NOS activity after treatment with guanabenz. Moreover, gel filtration of the guanabenz-treated sample did not restore activity. The per cent residual specific activity was 27% before filtration and 31% after gel filtration. In experiments for which data are not presented, the addition of oxyhemoglobin, which would react with NO, had no effect on the inactivation.

Guanabenz also caused the time-, concentration-, and NADPH-dependent inactivation of NOS activity present in rat brain cytosol with an apparent Kᵢ and Kᵢ₅₀ of 111 μM and 0.495 min⁻¹, respectively (data not shown). The presence of L-arginine, but not D-arginine, protected from this inactivation. The gel filtration of the inactivated sample did not restore activity of the brain cytosol (fig. 3).

**Effect of Guanabenz on NOS in Vivo.** Guanabenz (5.0 mg/kg/day) caused a decrease of approximately one-half in the rat penile NOS activity after 4 days of treatment similar to that found for N⁶-nitro-L-arginine (5 mg/kg/day), a known inhibitor of NOS (fig. 4A). One day of treatment with guanabenz had no effect on NOS activity (fig. 4A) even though this was of sufficient duration for the effects of N⁶-nitro-L-arginine (data not shown). In comparison, guanabenz caused a decrease of approximately one-quarter in the NOS activity present in brain cytosol of the treated rats (fig. 4B). The inhibition of brain NOS by N⁶-nitro-L-arginine was approximately 50% (fig. 4B), consistent with that reported previously (Dwyer et al., 1991), as well as closely matching the inhibition found in penile samples.

We focused on the inhibition of penile NOS activity. The inhibition by guanabenz after a 4-day treatment was dose dependent with observable effects down to 0.5 mg/kg/day (fig. 5). Moreover, the activity loss caused by guanabenz (5 mg/kg/day) was found to be concomitant with a decrease of approximately one-half in the level of immunodetectable NOS protein in the supernatant fraction prepared from the penis, as visualized by Western blot with the use of a monoclonal antibody (fig. 6). There were no detectable losses in the immunodetectable amount of NOS protein after inhibition with N⁶-nitro-L-arginine (fig. 6). There was no loss of immunodetectable NOS when penile cytosol was treated with guanabenz in vitro (fig. 7), under conditions where approximately 75% of the activity was lost. These results suggest that the inactivated form(s) of NOS under the in vitro conditions maintain the epitope structure for binding to the antibodies and also has not received extensive degradative metabolism to alter the molecular size of the protein.

**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; and ●, 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 described in Materials and Methods. The values are the mean and standard deviation (N = 3).

FIG. 2. Time-dependent inactivation of NOS activity in penile cytosolic fraction by guanabenz in vitro.

FIG. 3. Gel filtration of guanabenz-treated brain cytosolic fraction.

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).

FIG. 4. Time-dependent inhibition of NOS activity in penile (A) or brain (B) cytosolic fractions by guanabenz, in vivo.

A. Control, day 1A
Guanabenz, 1 day
Guanabenz, 4 days
NG-Nitro-L-arginine, 4 days

B. Control, day 4
Guanabenz, 4 days
NG-Nitro-L-arginine, 4 days

Residual Activity (% of control)

Guanabenz (mg/kg/day)

FIG. 5. Dose-dependent inhibition of penile NOS activity by guanabenz, in vivo.

The rats were treated for 4 days at the indicated doses of guanabenz as described in Materials and Methods. The values are the mean and standard deviation (N = 3).

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 Lubeskie, 1996). The N⁵-methyl-L-arginine is also a metabolism-based inactivator of the neuronal NOS with apparent Kᵢ and kᵢₑ values of 2 μM and 0.022 min⁻¹, respectively (Feldman et al., 1993; Reif and McCreedy, 1995). Other guanidine-containing compounds, such as aminoguanidine (Wolff and Lubeskie, 1995) and diaminoguanidine (Wolff and Lubeskie, 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 Lubeskie, 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 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 effects 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.

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